Vol. LXVI, No. 3 June, 1934

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

ELECTROKINETIC STUDIES OF MARINE OVA[1]

II. CUMINGIA TELLINOIDES, ASTERIAS FORBESII, ECHINARACHNIUS PARMA, NEREIS LIMBATA AND CEREBRATULUS LACTEUS

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In this study, an attempt was made to compare the cataphoretic potential of five types of marine eggs with that already described for sea-urchin eggs. The eggs chosen were those of the clam *Cumingia*, the starfish *Asterias*, the sand dollar *Echinarachnius*, the polychæte worm *Nereis*, and the nemertine *Cerebratulus*.

The method of measurement was the same as that described in the preceding paper (Dan, 1933). However, in this study the greater size of these eggs (except *Cumingia*) made necessary a slight modification in the procedure. The difficulty is that when, according to standard procedure, one tries to focus on an egg which is in the layer one-fifth of the distance across the chamber, the periphery of the egg comes so close to the wall that the latter exerts an influence on the movement of the egg. Strictly speaking, the egg is not free from this effect under any circumstances, but in case it lies sufficiently far from the wall, the effect becomes negligible. Therefore, I focussed to the middle layer of the chamber and studied the eggs in that layer. The effect of the electro-endosmotic current can be computed, as was described in the previous paper, from the graph obtained for this particular chamber. Another modification is the use of a lower magnification. *Nereis* eggs are very much heavier than the other types studied, and they therefore fall with a greater speed. As for *Asterias*, *Echinarachnius* and *Cerebratulus* eggs, even though their speed is not so great, their size is much larger than sea-urchin eggs and it is more convenient to observe them under a lower

[1] This paper and the preceding one of this series constitute a thesis presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

magnification. As a result, the accuracy of the data obtained in this way is not as great as that obtained by using a higher magnification, but it is, to be sure, good enough to determine the correct order of magnitude. When a lower magnification is used, the values obtained happen to be much smaller than those for *Arbacia* and *Cumingia* eggs, for which a higher magnification was adopted. This is, however, not an error due to the adoption of the lower magnification, for when *Arbacia* eggs with jelly are studied under similar conditions with low magnification, a charge of — 33.0 millivolts is obtained, and this value differs only 1 millivolt in absolute magnitude from the value obtained previously.

Cumingia tellinoides

Unfertilized Eggs with Jelly.—Freshly collected animals, after being kept dry for a while, were transferred to Stender dishes, one animal in each dish. After several females had shed eggs, these eggs were put together and the potential was measured. Some of the results are given in Table I. The average value of 23 measurements is — 34.1 millivolts.

TABLE I

Surface charge of unfertilized Cumingia eggs with jelly. Dielectric constant is taken as 80; viscosity is corrected to 25° C. (see text).

Potential gradient	Horizontal shift in 2.5 seconds	Values corrected for endosmosis and viscosity	Horizontal shift per second	Zeta potential
volt/cm.	*micra*		*micra*	*mv.*
	42.9	43.3	17.3	−34.0
	39.4	39.8	15.9	−31.1
	41.1	41.5	16.6	−32.6
6.7	47.0	47.4	19.0	−37.4
	41.6	42.0	16.8	−33.1
	41.6	42.0	16.8	−33.1
	45.8	46.2	18.5	−36.4
				Av. −34.0

In Table I, the dielectric constant of sea water is assumed to be 80, *i.e.*, that of distilled water. There are no measurements of the dielectric constant of sea water because of technical difficulties resulting from its high conductivity. As for the viscosity, the data for distilled water are used [2] and the cataphoretic speed is corrected to 25° C. to facilitate

[2] There is a measurement of viscosity of sea water by Krümmel and Ruppin (1905). If their figures are taken, the absolute magnitude of the zeta potential may become greater by 6–7 per cent. But, since the zeta potential itself can be only a relative measure in so far as our present knowledge goes, the data for distilled water are adopted in conformity with the previous work.

the comparison of these figures with those published in the preceding paper (Dan, 1933). The effect of temperature on the zeta potential itself is considered to be negligible within the range of temperature variation in this experiment (20°–24° C.) (Compare Burton, 1906).

Unfertilized Eggs without Jelly.—Eggs were secured in the same way as was described; then they were strained through bolting silk in order to remove the jelly. The result of 169 measurements gives the value of — 28.8 millivolts, the standard error being ± 0.46.

Asterias forbesii

Unfertilized Eggs with Jelly.—Eggs were obtained by cutting ovaries out of the body of a female and allowing mature eggs to stream out freely into the sea water. After fifteen or twenty minutes, the measurement was started. Eighty-two such measurements give an average value of — 19.0 ± 1.04 millivolts.

It may be noticed that the standard error is much greater here due to the adoption of a lower magnification. However, this is due mostly to the fluctuation involved in the process of free-hand tracing, as was discussed in the previous paper. This is shown by the fact that when eggs are cytolyzed and their speed of fall is thereby reduced, the measurement becomes relatively more accurate and a lower figure for the standard error can again be obtained, as will be seen later.

Unfertilized Eggs without Jelly.—Eggs, secured by the method which was described in the preceding section, were shaken vigorously so that the jelly would be removed. After shaking, samples were taken and were examined in Chinese ink suspension to determine whether or not the removal of the jelly was complete. Ninety-nine measurements were made, giving — 19.9 ± 1.01 millivolts as the average value. This figure is practically identical with that of eggs with jelly. In *Arbacia* and *Cumingia*, as well as in *Echinarachnius* (see below), the jelly shows a much higher absolute cataphoretic potential than the eggs. In so far as the data accumulated thus far are concerned, the *Asterias* egg is the only one in which the jelly has the same charge as the egg surface.

Eggs Killed by Heat.—There have been several studies concerning the effect of death upon the surface charge of cells. As early as 1906, Cernovodeanu and Henri discovered that bacteria retained their original charge materially unchanged, even after death of the cells by heat. Russ (1909) observed a similar fact with tubercle bacilli in urine. More recently Winslow et al. (1923) confirmed the earlier finding by using a more elaborate technique. Beside these facts, Abramson (1931) reported that "ghosts" of hæmolysed blood cells travelled with the identi-

cal cataphoretic velocity as that of intact cells. These observations are of great interest, but since they are concerned with rather simple and more or less quiescent materials such as bacteria or blood cells, it is very important to discover how more active cells would behave under similar circumstances.

Abramson (1928) made an observation in this direction. In his study of polymorphonuclear leucocytes, he compared the potential of active cells with that of degenerating cells. The latter were spherical in shape and non-amœboid. However, no difference was found between the speed of active and degenerating cells. He also pointed out that if human white cells were kept on ice for two days, there was no change

TABLE II

Surface charge of Asterias eggs (without jelly) killed by subjecting them to 40° C. for 5 minutes. Dielectric constant is taken as 80; viscosity is corrected to 25° C.

Potential gradient	Horizontal shift in 2.5 seconds	Values corrected for endosmosis and viscosity	Horizontal shift per second	Zeta potential
volt/cm.	*micra*		*micra*	*mv.*
	31.5	25.9	10.3	−20.2
	31.5	25.9	10.3	−20.2
	25.7	20.1	8.0	−15.7
	31.5	25.9	10.3	−20.2
6.7	36.2	29.4	11.8	−23.0
	24.5	18.9	7.6	−14.9
	29.1	23.5	9.4	−18.4
	33.9	28.3	11.3	−22.1
	31.5	25.9	10.3	−20.2
				Av. −19.4

in the cataphoretic speed. With these facts in mind, the following experiment was tried on *Asterias* eggs.

Eggs were obtained in the same manner as before and the jelly was shaken off. Then the eggs were subjected to 40° C. for 5 minutes. At the end of this period, all the eggs were completely cytolyzed, and the average diameter, which was 146.8 μ before the treatment, now measured 174.6 μ. The cytolysis was so complete that there was no chance for any eggs to survive the treatment. Some of the results are given in Table II. The final figure from 85 measurements is -19.0 ± 0.49 millivolts. It is surprising that the value for eggs killed by heat is identical with that of normal living eggs, in spite of the fact that the diameter and even the appearance of the cell has been so much changed by cytolysis.

Echinarachnius parma

Eggs were removed from females by cutting the tests open and putting ripe ovaries in an ample amount of sea water. Eggs were washed carefully. From 23 measurements, the average value for the potential of eggs with jelly is found to be — 31.6 millivolts. Occasionally a piece of jelly which had been detached from an egg cell was found. In such a case, even though the jelly itself is invisible because of its transparency, its presence can be detected by the pigment granules which are scattered through it. If a piece of this sort was selected and its cataphoretic speed measured, it was found to move with about the same speed as intact jelly still attached to the surface of the cell.

In this form it is rather hard to get eggs free from jelly, because of the delicacy of the egg cells, so that it was never certain whether or not the removal of jelly was complete. However, eggs which were, so far as could be determined, without jelly, moved about two-thirds as fast as those with jelly. This fact may indicate that the potential of the naked surfaces of eggs falls somewhere around — 20.0 millivolts. In one experiment tissue cells were involved in the egg suspension, and these tissue cells showed a speed of the same order of magnitude as egg cells without jelly.

Nereis limbata (*Unfertilized Eggs*)

The material was collected in the evening and was sometimes used immediately. Often, however, it was experimented upon early the following morning. The body of a female was cut open, the eggs were washed carefully, and they were then quickly brought into the chamber. Because of the great speed of fall of these eggs, the measurements are not as accurate as the preceding ones. The mean value of 76 measurements is — 9.7 millivolts.

Cerebratulus lacteus (*Unfertilized Eggs*)

Eggs were secured by cutting open the body of a female. Some eggs were irregular in shape immediately after being taken out of the body, but later they became more spherical. The experiments were performed only after this state was reached.

As is well known, the *Cerebratulus* egg is enclosed in a huge chorion. This chorionic membrane has a certain degree of rigidity, but it is not difficult to remove it if desired. The large space within the membrane seems to be filled with some sort of a viscous organic fluid, for when seen from the side, the egg cell is always suspended, whereas if it is

taken out into the sea water, it sinks very rapidly. Therefore in this case we must take account of the fact that the system we are dealing with is a composite one, consisting of three component parts: chorionic membrane, chorionic fluid, and egg cell. This is a very important point to keep in mind.

Surface Charge of the Chorion.—First, a chorion containing an egg was put into the cataphoresis chamber and its behavior in the electric current was observed. The chorion of *Cerebratulus* has an ovoidal form. Its long axis measures about 300 μ and the short axis about 200 μ. Therefore, taking 250 μ as the average diameter, for the purpose of a rough calculation, the strength of the endosmotic current which is originated by the wall of the cataphoresis chamber and is acting on the chorion is calculated. If this is done, since the observed speed is the addition of the true cataphoretic speed and the shift caused by the electroendosmotic flow, the former can easily be known. As a result of this calculation, it was found that the speed of migration of the chorion toward the anode far exceeded that which might be attributed to the electroendosmotic current acting on the chorion. Thus it is certain that the surface of the chorion is negatively charged.

Migration of the Egg Cell within the Chorion.—During the course of various experiments, chorions were sometimes found which, for some reason, were attached to the wall of the chamber. In this case, the chorion was incapable of free movement, and the behavior of the egg cell suspended in the chorionic fluid could be studied. The striking thing which was found in these cases was the migration of the egg cell toward the cathode (it is thus apparently positive). The speed of this cathodic migration was very small and, so far, no reliable figure has been obtained. After the egg reached one end of the chorion, it became somewhat flattened against the membrane, while the membrane itself was made to bulge out under the pressure of the egg. If the current was reversed, the egg regained its spherical form, changed its direction of migration, and travelled across the chorionic space very slowly. When it came to the other end, the same flattening occurred. This could be repeated many times with the same egg by reversing the current. The accompanying camera lucida drawing (Fig. 1) illustrates the phenomenon above described. This sketch, however, does not represent the highest degree of flattening, for the breaking of the current to permit drawing allowed the egg to resume its spherical form.

Movement of the Egg Cell in Sea Water.—In other experiments, the chorion was removed by straining the egg through bolting silk. The potential on the naked surface of the egg cell was measured in the same way as was done in the case of *Asterias* and other eggs. The mean

value of 16 readings was found to be — 1.4 millivolts. Unfortunately 16 measurements is a very small number. *Cerebratulus* material is very difficult to obtain at Woods Hole, and only one worm was available during the entire summer of 1932 and none in 1933. As a result of the small number of determinations and the relatively high standard error, the value arrived at is not sufficiently reliable to establish the negativity of the potential. A greater number of determinations might very well give a small positive value, which would explain the slow cathodic migration of the eggs mentioned above. At any rate, for the present, it can at least be said with certainty that the charge on *Cerebratulus* eggs is in the vicinity of zero.

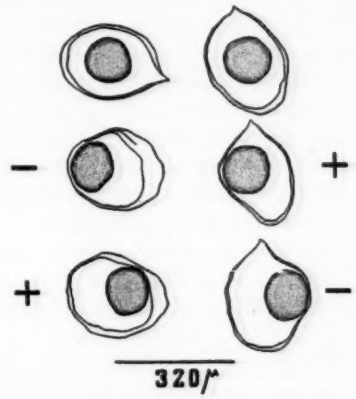

FIG. 1. Eggs of *Cerebratulus lacteus* within chorions. Each column represents the behavior of a single egg before and after the application of the electric current. Note the cathodic migration of the cells within their chorions.

In spite of the drawback in the measurement above stated, this egg indicates many interesting features. The first point to be noted is the peculiar fact that *Cerebratulus* eggs show so small a potential in such an alkaline medium as sea water (pH 8.2). Of course, there are several kinds of bacteria known to have a very low potential (in absolute magnitude) through a wide range of hydrogen ion concentration (Mudd and Joffe, 1933). Also there are several observations of the acquisition of zero or even a positive potential by bacteria in high pH values. The paper of Winslow et al. quoted above (1923) serves as one example of this phenomenon. Moreover, Vlès and Nouel (1922) and later Vlès (1924), in their experiments on agglutination of sea-urchin eggs at various pH values, found that there was a secondary agglutination point

at an extremely high pH region. This might indicate, though indirectly, that sea-urchin eggs (*Paracentrotus lividus* Lk.) have a secondary isoelectric point in an extremely alkaline medium. At present, however, experimental data are too scanty to warrant speculation concerning the *Cerebratulus* egg. It is hoped that the investigation can be extended in this direction in the near future.

The second point of interest is the migration of these eggs within the chorion. This naturally leads to the question whether or not other eggs which are surrounded by transparent jelly behave in a similar way in an electric field. Therefore a qualitative observation was undertaken by using the eggs of *Echinarachnius*, *Asterias*, and *Arbacia*. The experiment consisted of sending an electric current through an India ink suspension containing eggs under cover slips. As soon as the circuit was made, the existence of a strong electroendosmotic current was revealed by the fact that all the ink particles on the cathodal side of the jelly were completely washed away from its vicinity. This is due to a current of water flowing through the jelly from the anodal side to the cathodal side, as is to be expected from the negative charge on the jelly noted above. However, in spite of this striking change in the surrounding medium, no shift was observed in the relative positions of the jelly and the egg cell. This is probably due to the difference in the physical nature of the chorionic fluid of *Cerebratulus* and that of the jelly layers of other eggs. On the other hand, Freundlich and Abramson (1927) discovered that red-blood cells migrate as fast cataphoretically in 1 per cent gelatine gel as in a sol of the same concentration. However, until the exact physical nature of the jelly is known, no conclusion can be drawn.

As for the flattening of the cell against the chorionic wall, Mazia (1933) reported a strikingly similar behavior in frog eggs (with jelly), and he also found that frog eggs migrated toward the cathode even after the jelly was removed by KCN. Another similar phenomenon was observed long ago by Carlgren (1900) in the parthenogonidia of *Volvox*, although the direction of the migration was opposite, namely toward the anode. Since I have not had the opportunity to study this material, I venture no opinion concerning it. At any rate, it is remarkable that a cell which is surrounded by a certain structure can migrate and flatten itself against the wall of its containing envelope.

Bungenberg de Jong (1932) has studied a somewhat similar phenomenon in non-living systems, and it is instructive to compare the analogous behavior of living and non-living systems.

Here I acknowledge my great indebtedness to Dr. L. V. Heilbrunn under whose direction this work was completed.

SUMMARY

1. By means of the method described in an earlier paper and a slight modification of it, the surface charge of five kinds of marine ova was measured.

2. *Cumingia* eggs with jelly show a charge of —34.1 millivolts (23 measurements); eggs without jelly show a charge of -28.8 ± 0.46 millivolts.

3. *Asterias* eggs with jelly have a charge of -19.0 ± 1.04 millivolts; those without jelly -19.9 ± 1.01 millivolts; and eggs without jelly which were killed by heat have a charge of -19.0 ± 0.49 millivolts.

4. *Echinarachnius* eggs with jelly have a charge of —31.6 millivolts (23 measurements), and eggs without jelly seem to have a charge of about —20.0 millivolts.

5. *Nereis* eggs, according to less accurate data, show a charge of about —10.0 millivolts.

6. In the case of the *Cerebratulus* egg, the negative charge of the chorionic membrane was first ascertained; next the cathodic migration of the egg cell within the chorion was observed; and finally the surface charge of the cell in sea water was demonstrated to be in the vicinity of zero.

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THE HYALINE ZONE OF THE CENTRIFUGED EGG OF CUMINGIA

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The egg of the lamellibranch mollusk, *Cumingia tellinoides,* has been extensively used as material for studies with the centrifuge, beginning with the classical experiments of Morgan (1908*a,* 1908*b,* 1909, 1910). The results of these investigations indicated that the visible granular substances of the protoplasm, displaceable with centrifugal force, bore no necessary relation to the polarity, symmetry, or organ formation of the developing embryo. Since these embryological studies were made, *Cumingia* eggs have been centrifuged by Heilbrunn to study the changes in protoplasmic viscosity during maturation, fertilization, and cleavage (1920, 1921, 1925); to determine the effects of temperature on the viscosity of protoplasm (1924*a,* 1924*b*); and for measurements of the absolute viscosity of protoplasm (1926). E. N. Harvey has centrifuged these eggs to measure the tension at the surface (1931*a*), and as material for microscope-centrifuge studies (1931*b,* 1932). They were also centrifuged by Costello (1932), to determine the rôle of the granular constituents of the protoplasm in the surface precipitation reaction.

Morgan (1910) describes the appearance of the centrifuged unfertilized egg of *Cumingia* as follows: "When the egg is centrifuged the pigment and yolk are carried to the outer pole, the oil to the inner. Between the two lies a band of clear protoplasm." This description is based on sections as well as on the appearance of the living egg. Of the displaced granular constituents, it is the yolk alone, according to Morgan, which appears in the sections, the pigment and oil being dissolved by the reagents. The broad clear zone is figured as containing finely granular, stainable protoplasm. Morgan (1908*a,* 1908*b,* 1909) had previously described in less detail the three zones produced by centrifuging.

Morgan (1927, p. 501) has amplified the above description as follows: "After centrifuging, the egg shows four zones, similar to those in centrifuged eggs of the sea-urchin. There is (1) a large oil cap; (2) a broad, clear zone; (3) a small yolk field; and (4) a pink, pigmented region opposite to the oil cap."

All other workers have described but three zones. Heilbrunn (1920) says: "To one pole pass the presumably lighter oil globules, to the opposite pole the heavier pigment." Harvey (1931*b*) says: ". . . the oil is stratified in two-thirds the time necessary to stratify the yolk." These workers observed only living material.

The question naturally arises as to whether there are three zones and two types of granules in the centrifuged egg of *Cumingia*, or four zones and three types of granules (assuming the hyaline zone in both cases to be non-granular). It is quite evident that Harvey and Heilbrunn have observed but one type of granule at the centrifugal pole of the egg, and that in their descriptions yolk and pigment are synonymous. It is well known that the pigmentation of these granules may vary from deep pink to an almost colorless condition in different lots of eggs.

Unfortunately Morgan does not give the centrifugal force developed by the Bausch and Lomb hand centrifuge nor by the water centrifuge which he used in his experiments. From his figures, however, we may be certain that the forces used were small. Heilbrunn (1924*a*) used a force of 311 times gravity and stratified the eggs in 90 seconds or less. Heilbrunn (1925, 1926) used a force of 4,968 times gravity, and stratified the eggs in 5 seconds or less. Harvey (1931*a*) used a force of 6,595 times gravity to pull the eggs apart.

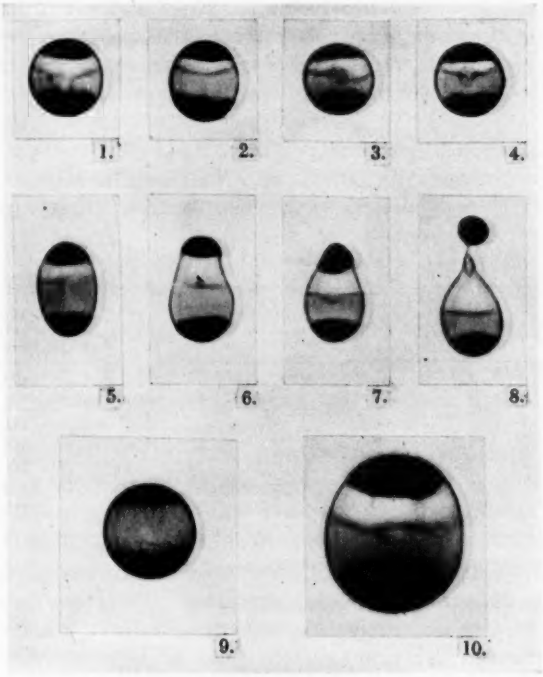
I have been interested in the application of great centrifugal forces for long periods of time. If the eggs are centrifuged for 10 to 20 minutes with a force of about 6,000 times gravity, in hæmatocrit tubes containing a centrifugal layer of isotonic sucrose, a stratification of the egg is produced markedly different from that described above. (See Photographs 1–10). There are visible in the living centrifuged egg the following zones: (1) an oil cap; (2) a clear hyaline zone containing no visible granules; (3) a broad zone of very small colorless granules; and (4) a zone of pink, pigmented granules. Zones (2) and (3) have been produced by separating out the finely granular material from the hyaline protoplasm in the broad, clear zone described by Morgan. The spindle may lie at any angle with respect to the stratification. The spindle generally lies in the zone of small colorless granules in the more strongly centrifuged eggs, or at the border of the hyaline zone and the zone of small granules in the less strongly centrifuged eggs.

Eggs centrifuged at any time after fertilization before the second cleavage may be stratified by prolonged centrifuging to give these same zones, though the duration of time of centrifuging to produce a given end-point varies with the phase of mitosis (see Heilbrunn, 1921).

If the eggs are centrifuged for 30 seconds with a force of 6,000 times gravity, or for a correspondingly longer time at lower forces, only three

zones are produced. These are: (1) the oil cap; (2) the broad, clear zone; and (3) the pigmented zone.

The increase in height of the true hyaline zone formed by the movement centrifugalwards of the granules of the broad clear zone may be expressed as a function of the centrifuging time (for a force of about 6,000 times gravity). (See Table I.)



EXPLANATION OF PHOTOGRAPHS

All photographs are of living unfertilized eggs, arranged with the oil cap uppermost in each case.

PHOTOGRAPHS 1 AND 2. Egg centrifuged 3 minutes, photographed 14 minutes later.

PHOTOGRAPHS 3 AND 4. Egg centrifuged 5 minutes, photographed 12 minutes later. Maturation spindle visible.

PHOTOGRAPH 5. Egg centrifuged 15 minutes, photographed 5 minutes later.

PHOTOGRAPH 6. Egg centrifuged 20 minutes, photographed 4 minutes later.

PHOTOGRAPH 7. Egg centrifuged 30 minutes, photographed 1 minute later.

PHOTOGRAPH 8. Same. The oil cap is pulling off as a spherical fragment.

PHOTOGRAPH 9. Egg centrifuged 1 minute, photographed 30 minutes later.

PHOTOGRAPH 10. Egg centrifuged 10 minutes, photographed 9 minutes later.

With prolonged centrifuging (30 minutes or longer), the oil cap separates as a sphere from the rest of the egg. (See Harvey, 1931*a*.)

In sections of centrifuged eggs fixed by the Champy-Kull technique, sectioned, and stained with Heidenhain's hæmatoxylin, the same four zones of stratification are found. The hyaline zone has coagulated to give a finely granular appearance. The small colorless granules remain, but do not stain intensely by this procedure. The oil globules are preserved, and appear as intensely black spheres at the centripetal pole of the egg. Since the term yolk has not been applied to any specific chemical substance in the eggs of marine invertebrates, the exact terminology to be applied to the pigmented granules seems of little importance.

Heilbrunn (1926) obtained a value of 0.043 poises for the absolute viscosity of *Cumingia* egg protoplasm. This value is based on the assumption that the pigmented granules were moving through hyaline or

TABLE I

Centrifuging time in minutes	Height of hyaline zone in fractions of egg axis	Height of zone of small granules in fractions of egg axis
1/2	0	1/2
1	0	1/2
3	1/16	7/16
5	1/8	3/8
10	1/4	1/4
20	3/10 (egg pear-shaped)	3/10
30	2/5 (oil cap pulling off)	1/5

granule-free protoplasm. Actually, they were moving through the finely granular protoplasm of the broad clear zone. Therefore the absolute viscosity of the hyaline protoplasm would be somewhat lower than this figure. However, Harvey (1931*b*) gives a value for the average diameter of the pigment granules as 0.5 μ. My own measurements of granules in fixed preparations tend to verify this value, although measurements of such small particles are never very certain. Neglecting the Cunningham correction, this would give the suspension of the broad clear zone a viscosity of 0.118 instead of 0.043 poises. Several equations have been derived for estimating the viscosity of the dispersion medium when the viscosity of the suspension and the volume concentration of the suspended particles are known. For the egg of *Cumingia*, the volume of the zone occupied by the small colorless granules is about equal to the volume of the true hyaline zone. If this zone consisted of packed spherical granules of uniform diameter, they would occupy ap-

proximately 74 per cent of the total volume of this zone. However, there is probably considerable variation in granule size, and it is not certain how much packing occurs. As an approximation we may take the relative volume occupied by the granules in the broad clear zone as 37 per cent. The concentration of granules at which the viscosity is infinity is assumed to be 74 per cent, though this is probably too high. The equations for the viscosity of a suspension, with the values obtained for the viscosity of the hyaline protoplasm of the *Cumingia* egg, by the use of each, are listed below:

(1) $\eta_s = \eta \frac{1 + 0.5f}{(1 - f)^4}$ (Einstein, from Kunitz, 1926) $\eta = 0.016$

(2) $\eta_s = \eta(1 + 4.5f)$ (Hatschek, 1910) $\eta = 0.044$

(3) $\eta_s = \eta \frac{1}{1 - af}$ (Hess, 1920)

(3a) $\eta_s = \eta \frac{1}{1 - \sqrt[3]{f}}$ (Hatschek, 1911) $\eta = 0.033$

(4) $\eta_s = \eta \frac{1}{(1 - f - 3/2f^{5/3})^{5/2}}$ (Smoluchowski, 1916) $\eta = 0.008$

(5) $\eta_s = \eta \frac{1}{\left(1 - \frac{f}{f_\infty}\right)}$ (Bingham and Durham, 1911) $\eta = 0.059$

where η_s is the viscosity of the suspension (0.118), η is the viscosity of the dispersion medium, f is the ratio of the volume of suspended particles to the total volume of the suspension (37 per cent), f_∞ is the value of f at which viscosity becomes infinite (74 per cent), and a is a constant.

As Kunitz points out, equation (1) is purely empirical. Equation (2) may be applied in cases where f is large. Equation (3), involving the constant a, cannot be applied except by assuming

$$a = \frac{1}{\sqrt[3]{f^2}} (= 1.94),$$

which makes this equation identical with one previously derived by Hatschek (3 *a*) for very concentrated suspensions (> 50 per cent) of deformable particles. Hess has applied this equation to suspensions of both low and high concentration. Equations (4) and (5) are also empirical. It is not possible to state at present which of these equations applies best to this particular case, so the values calculated are indicative only of the order of magnitude of the viscosity of the hyaline proto-

plasm. Obviously, any value lower than the viscosity of water (0.01 poises) is an impossible figure.

Completely stratified *Cumingia* eggs were crushed in the manner described by Costello (1932) and the presence or absence of a surface precipitation reaction observed for the different zones of stratification. This was done to determine whether or not the small colorless granules are directly involved in the surface precipitation reaction, as Heilbrunn has previously shown for the pigment granules of the *Arbacia* egg. It was usually possible to obtain a surface precipitation reaction from the heavy pole of the egg, if the egg were gently ruptured. From protoplasm extruded at the light pole, however, a definite membrane was never obtained. Whether this is due to the greater fluidity of the protoplasm at this pole, or due to the absence of a particular type of granule, could not be determined. Since the granules of the zone of small colorless granules are about at the limits of microscopical vision (0.2 μ or less in diameter), it was not possible to determine whether or not they disintegrated during the formation of the surface precipitation membrane at the heavy pole of the egg.

Considering the results outlined above, Morgan's (1910) analysis of the relation between organ-forming substances and embryonic localization in the egg of *Cumingia* is somewhat incomplete, since it does not preclude the possibility of almost normal distribution of the small colorless granules of the broad clear zone to all of the early blastomeres. (The small colorless granules are of such a size that they could occupy the interspaces between the packed pigment granules or oil in the eggs centrifuged with the forces used by Morgan.) However, considering the results of the centrifuge experiments on other eggs, it seems improbable that the small colorless granules are any more morphogenic than the other types of granules. (But compare the results of Conklin, 1931, obtained by prolonged centrifuging of the egg of *Styela*.)

Summary

1. The zones of stratification of the centrifuged egg of *Cumingia* have been redescribed.

2. The relations of the presence of granules in the "broad clear zone" to previous work on (*a*) absolute protoplasmic viscosity, (*b*) the surface precipitation reaction, and (*c*) embryonic localization have been discussed.

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THE EFFECT OF ANESTHETICS ON THE SURFACE PRECIPITATION REACTION

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Living substance is unique in its capacity to respond to sudden changes in its environment. This innate irritability may, however, be lost temporarily under the influence of various anesthetics. In any general interpretation of irritability, the phenomenon of anesthesia must be carefully considered, and, indeed, if we could explain anesthesia we would be well on our way toward an interpretation of what actually happens when protoplasm is aroused to activity. It is not surprising, therefore, that so many authors have attempted to solve the riddle of anesthesia. Much of this work is summarized in Winterstein's excellent monograph (1926). A more recent review is that of Henderson (1930). The paper of Bancroft and Richter (1931) may also be consulted.

Some years ago, I began to gather data in favor of the view that stimulating agents cause a gelation of the protoplasm and that the primary action of anesthetics was to prevent such a gelation. This point of view was stressed in a monograph published in 1928. Facts were presented to show that stimulation does actually cause a very sharp increase in protoplasmic viscosity and that such general stimulating agents as the electric current or ultraviolet radiation do indeed stiffen the protoplasmic fluid. An attempt was also made to interpret the gelation involved in stimulation. Response was thought to be due to a type of clotting, the mechanism of which was essentially similar to that involved in the injury reaction which occurs when a cell is torn or broken. As the protoplasm begins to emerge from such a torn cell, a peculiar and distinctive type of precipitation reaction occurs. This I call the surface precipitation reaction, or s.p.r. A new film or membrane appears, and this membrane formation at the border of the exuding droplet may be accompanied by the formation of numerous new films within the droplet. As a result, many small vacuoles frequently appear, and this vacuolization may extend throughout the entire cell. Such a complete vacuolization usually leads to death, which is frequently the result of overstimulation. The gelation which occurs after normal stimulation is

thought to involve a reaction of the same general nature as the surface precipitation reaction, and it may be interpreted in terms of this reaction. In the present discussion, I shall limit myself to the simple concept outlined above, although the actual events may be much more complicated (compare Heilbrunn and Daugherty, 1933).

If, then, stimulation causes clotting of protoplasm, and if, moreover, this clotting is essentially an internal surface precipitation reaction, then it should follow, first, that anesthetics prevent clotting or gelation in the cell interior, and, second, that they should have an inhibiting effect on the s.p.r. In earlier work, I was able to show that ether and other fat solvents do actually exert a liquefying action on protoplasm, and do prevent the gelation which appears on stimulation (for details consult Heilbrunn, 1928). This much is in accord with theory. But, if the gelation which accompanies stimulation is fundamentally the same type of reaction as the s.p.r., then it should be possible to demonstrate an effect of fat solvents on the s.p.r. as it occurs when a cell is torn or broken.

Earlier attempts in this direction met with failure. When sea-urchin eggs were torn or broken in the presence of ether dissolved in anesthetic concentration in the surrounding sea water, the normal type of precipitation reaction occurred, and there was not the slightest evidence of any retarding effect of the ether. Various fat-solvent anesthetics were tried, and in no case was the s.p.r. retarded or prevented in their presence. Observations of this sort constituted a serious stumbling block to the point of view which I have been advocating.

It must be remembered, however, that when a sea-urchin egg is torn or broken in sea water, the surface precipitation reaction occurs in the presence of an abundance of free calcium. On the other hand, if an s.p.r. were to occur within the cell, the reaction would presumably take place in the presence of a very small amount of calcium, for there is good reason to believe that within the cell most of the calcium is bound chemically. In view of the fact that calcium plays such an essential rôle in the s.p.r., this is an important difference.

During the past summer, in the course of some experiments with the magnesium ion, I found that ether prevented the surface precipitation reaction which this ion may produce. It will be shown later that magnesium acts like calcium, except that its effect is much less potent. Thus, a dilute solution of calcium acts like a much stronger solution of magnesium. It was decided, therefore, to test the effect of ether on the s.p.r. in the presence of a very low concentration of the calcium ion. Under these conditions, it was indeed found that ether does prevent the s.p.r. Some of these observations will now be presented in detail.

Action of Ether on the S.P.R. in Arbacia

Only the simplest type of experiment was performed. In my original description of the s.p.r. in the *Arbacia* egg (Heilbrunn, 1927), it was shown that a trace of calcium was sufficient for the reaction. Thus if eggs are placed in isotonic sodium chloride solution and crushed, there is enough calcium carried over with the eggs to permit a reaction. If, however, eggs are washed once or twice in isotonic sodium chloride solution and then crushed, no reaction occurs.

Experiments were performed with solutions which contained 1000 parts of an isotonic sodium chloride solution (0.53 M) to one part of an isotonic calcium chloride solution (0.3 M). One drop of egg suspension was placed in 20 cc. of such a solution, and then after a minute a drop of the eggs was pipetted into a second dish also containing 20 cc. of the same solution. The eggs were then placed on a slide under a cover and crushed. The crushing was usually accomplished by sucking out fluid from the side of the cover slip with a piece of filter paper. When the pressure becomes great enough, the eggs break, the protoplasm streams out, and the small exuding droplet forms a membrane around itself. Pigment granules in the extraovate gradually disappear from view. There is thus a typical s.p.r. A peculiar phenomenon then follows in most of the eggs which are crushed in these solutions containing little calcium. Membrane elevation similar to that produced by the sperm in the fertilization reaction now occurs in that part of the egg which has remained intact. Starting from the rim of the extraovate, that is to say, from its junction with the uninjured protoplasm, the vitelline membrane slowly starts to lift away from the cytoplasm of the egg. This membrane elevation proceeds around the egg surface until the entire uninjured portion of the egg has a perfect fertilization membrane. It is believed that this observation of membrane elevation following injury is of real importance for the theory of membrane elevation, but I shall not discuss it further in this paper, except to point out that the phenomenon occurs only when the calcium concentration is low. In sea water there is no evidence of it.

If eggs are immersed as before in 1000 parts by volume of isotonic sodium chloride solution plus 1 part of isotonic calcium chloride solution, with now the addition of 2 or 3 cc. of ether per 100 cc. of solution, a different result is obtained. To prevent the evaporation of ether, the eggs are placed in glass-stoppered weighing bottles, but otherwise the treatment is the same. When the etherized eggs are crushed under a cover slip, no surface precipitation reaction occurs, and there is no subsequent membrane elevation. Usually the pigment granules emerging

from the crushed eggs remain intact and visible. The ether has thus interfered with the surface precipitation reaction, and has prevented both breakdown of pigment granules and formation of a new film or precipitation membrane. (Membrane elevation is also inhibited.)

Ether can thus prevent the s.p.r. in the *Arbacia* egg, but this antagonism occurs only when the calcium concentration is low. If the calcium concentration is doubled, that is to say, if the eggs are crushed in 500 parts of isotonic sodium chloride solution plus 1 part of isotonic calcium chloride solution, the effect of the ether is not so noticeable, and with slightly higher concentration of calcium, it disappears completely.

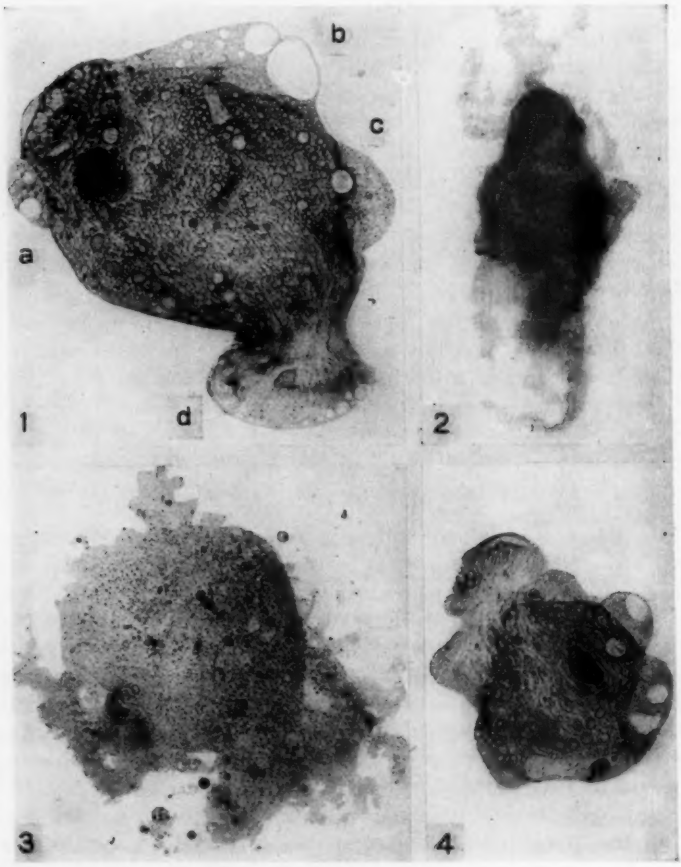
The action of ether on the s.p.r. may be demonstrated very simply by comparing the reaction in an isotonic sodium chloride solution with that which occurs in a similar solution containing 2 per cent ether (by volume). When a drop of egg suspension is added to 20 cc. of an isotonic sodium chloride solution, and the eggs are crushed, there is a clear-cut reaction involving both breakdown of pigment granules and formation of a new surface film. On the other hand, in the presence of ether, the reaction is inhibited. In performing this experiment, eggs should be tested within a few minutes after immersion into the solutions, for with increasing length of exposure to isotonic sodium chloride, the surface precipitation reaction becomes less distinct and the pigment granules tend to remain intact on escaping into the sodium chloride solution. A similar time effect can also be noted in the case of magnesium solutions.

Action of Ether on the S.P.R. in Stentor

The action of ether in suppressing the s.p.r. may be very clearly demonstrated for the protozoön *Stentor*. This form is ideal for a study of the reaction and when the cell membrane of the stentor is broken, a beautiful s.p.r. occurs. Through the kindness of Dr. Hetherington, I was able to secure an excellent culture of *Stentor cæruleus*, and numerous experiments were performed with it. Later other cultures were obtained in Philadelphia. In these the animals were reared in a dilute wheat infusion in pond water. Similar results were obtained with them.

When *Stentor* is placed on a slide, and a cover slip is pressed down over it so that the protoplasm is forced out through a rupture of the body wall, the emerging droplet immediately forms a membrane about itself. I know of no protozoön which gives a clearer reaction. Figure 1 is an unretouched photograph which shows the phenomenon clearly. Several droplets with their membranes appear at *a, b, c*. There is evi-

dently a sharp border between the protoplasm which has been pressed out of the cell and the surrounding medium. At *d* the protoplasm is moving out of the cell, and a membrane is just being formed. The s.p.r. in *Stentor* is also shown in Fig. 4, in which the background has been blocked out for purposes of contrast.



EXPLANATION OF PLATE I

FIGS. 1 AND 4. Surface precipitation reaction in *Stentor*.

FIGS. 2 AND 3. Absence of surface precipitation reaction in the presence of 1 per cent ether.

The magnification is × 80 for Figs. 1 and 3, and × 50 for Figs. 2 and 4.

If, now, stentors are immersed in 1 per cent ether in tap water (Woods Hole or Philadelphia) and crushed in the same manner as before, no s.p.r. occurs, and the protoplasmic granules stream through the solution. This is clearly shown in Figs. 2 and 3, unretouched photographs. In order to make certain that the effect is due to the ether and not to the tap water, control experiments were performed in which animals were crushed in tap water alone. In such tests, a perfect s.p.r. was obtained. It is evident, therefore, that in *Stentor*, as well as in *Arbacia*, ether exerts an inhibiting effect on the s.p.r. A concentration of ½ per cent ether is not as effective as 1 per cent ether in stopping the reaction. In 2 per cent ether, no typical s.p.r. occurs, but the protoplasmic granules from crushed specimens do not scatter through the solution as is the case when 1 per cent ether is used.

Results similar to those obtained with ether were noted when *Stentor* was immersed in 1 per cent butyl alcohol and crushed. Ethyl urethane, amyl alcohol, and acetone were also effective in preventing the s.p.r.

The results obtained indicate that ether (and other fat-solvent anesthetics) can exert an inhibiting effect on the s.p.r. This effect is more readily observed in *Stentor*, for it may be noted when ether is added to the solutions in which *Stentor* lives. On the other hand, in the case of sea-urchin eggs, in order to show the effect of ether, one must study its action in solutions which contain only a very small amount of calcium.

Moreover, in the case of sea-urchin eggs, when ether in relatively high concentration (3–4 per cent) is added to the sea water surrounding them, the eggs undergo a vacuolization reaction which is thought to be essentially an internal surface precipitation reaction. Thus, although under some conditions ether may inhibit the s.p.r., under other conditions it may actually initiate the reaction. The latter effect is believed to be due to the fact that ether releases free calcium into the egg interior. This free calcium then initiates the reaction even though ether is present.

It thus appears that ether (and presumably fat solvents generally) can both favor an s.p.r. and prevent it. We thus have a formal explanation of the queer fact that fat-solvent anesthetics are sometimes stimulants. In the case of the *Arbacia* egg, the gelating or stimulating effect of ether preponderates at higher concentrations of the fat solvent.

Relation of the Magnesium Ion to the S.P.R.

Most theories of anesthesia find the fact of magnesium anesthesia a stumbling block. They have no way of explaining it. Magnesium, it is true, may lower permeability, but its chief antagonist is calcium, which has a similar effect on permeability. This difficulty has bothered some of the adherents of the permeability doctrine, so much so that

Höber (1926) has been led to state that magnesium anesthesia is not a true anesthesia; for, says he, true anesthetics are all surface-active, they penetrate cells and paralyze all their functions reversibly, at least more or less, whereas magnesium acts primarily at cell junctions or synapses. In view of the fact that magnesium salts are perhaps the most widely used anesthetics for marine invertebrates, and have an anesthetic action on widely divergent organisms from amœba (Heilbrunn, 1932) to man, it is not so easy to dismiss them as anesthetics. Perhaps, after all, it is a question of definition. Personally, I doubt if magnesium is less of an anesthetic than ether.

How then shall we explain the action of the magnesium ion? In a recent note (Heilbrunn, 1932; see also Heilbrunn and Daugherty, 1932) it was shown that magnesium tends to liquefy the plasmagel of the amœba. This fact fits in well enough with the general theory of anesthesia outlined above, and it is also in accord with other evidence as yet unpublished which indicates that various types of anesthetics all liquefy the plasmagel of *Amœba proteus*. But there is another side to our theory, for stimulation is thought to involve a reaction related to the s.p.r. What effect then does the magnesium ion have on the s.p.r. and is it possible to relate such an effect to the known facts of anesthesia?

In my 1927 paper, the relation of magnesium to the s.p.r. was considered briefly and not very correctly. It was stated that magnesium was not a good substitute for calcium, and that when an s.p.r. occurred in magnesium solutions, it was due to a secondary process. As a matter of fact, magnesium may be substituted for calcium. Indeed, an excellent s.p.r. occurs in isotonic solutions of magnesium salts.

When *Arbacia* eggs are immersed in a solution of 0.3 M magnesium chloride, and are then crushed after a minute or two, there is a clear-cut reaction at the surface of the exuding droplet of protoplasmic fluid and an excellent membrane is formed. Moreover, the pigment granules break down, or at least some of them do. As a matter of fact, the behavior of the pigment granules in these eggs is very interesting. Typically, when a droplet of protoplasmic fluid begins to emerge from the torn surface of the egg, the pigment granules are at first intact in the exuded mass. Breakdown of granules then begins in a very limited region. The broken egg is dumbbell-shaped, consisting of a large sphere which is the original egg cell, and a smaller sphere, the exuded droplet or extraovate. Between the two there is a relatively narrow neck of material. It is in this neck, the original cortex of the egg in the injured area, that pigment-granule breakdown occurs. From this initial point of pigment-granule breakdown, a wave of reaction spreads in both directions into and across the original egg cell on the one side, and out into

the exuded droplet on the other. The phenomenon is very striking. As the pigment granules break down at any one point, they release a bright red color, and vacuoles come into view. At the next instant, neighboring granules go through the same process and the reaction is repeated across the cell. Often the wave of reaction goes completely across the original cell and the extraovate, so that all parts of the dumbbell are affected. The speed of the wave can readily be measured. Usually at room temperatures it takes approximately 15 seconds for the wave to pass to the farthest end of the cell. There is more or less variation in speed with different degrees of injury. Even so, fairly consistent values may be obtained. Thus, six tests showed 15, 14, 18, 14, 21, 15, 18 seconds. When eggs are crushed in isotonic calcium chloride solution, there is a similar wave of reaction across the cell. In this case, however, it appears to be more rapid, and the egg is usually traversed in about 5 seconds.[1] In calcium chloride solutions, breakdown cannot be seen to begin at the neck of the dumbbell, but occurs almost instantly in all of the exuded droplet. In solutions of magnesium chloride, the wave of reaction is often halted before it passes across the egg or before it crosses the extraovate. Thus, in eggs crushed in magnesium chloride solutions, one often sees intact pigment granules in the extraovate. Once the reaction wave has been halted, the pigment granules beyond its path seem to acquire a resistance to breakdown. This applies both to the granules within the egg proper and those in the extraovate.

When the s.p.r. is observed in sea water, the pigment granules in the extraovate all break down and there is also a wave of breakdown which passes across a fraction of the original egg. Usually the wave across the egg itself is halted before it proceeds very far.[2] If, now, the egg be crushed a second time, those pigment granules which resisted breakdown after the first injury lose their pigment and disappear. Eggs in magnesium chloride solutions behave differently. If they are crushed once, and if the wave of pigment-granule breakdown fails to include the whole egg, those granules beyond the path of the wave become resistant to breakdown. When the egg in magnesium solution is crushed a second time, the intact pigment granules do not break down, even though they float free in the surrounding medium. It is thus apparent that under the conditions described above, the pigment granules have acquired an immunity to breakdown.

It is possible that in this observation lies a clue to the nature of the anesthetic action of the magnesium ion. We can conceive of living

[1] These values for speed of reaction wave across the cell are for eggs crushed within a few minutes after immersion into the solutions. Following longer times of immersion, the waves move more slowly.

[2] This stoppage of the wave is apparently due to the presence of sodium ion.

protoplasm as being always in a state of unstable equilibrium between those factors which favor an s.p.r. and those which inhibit or reverse it. Then, once an s.p.r. occurs, it must, to some extent at least, be reversed before the protoplasm can become irritable again, so that normal stimulation in itself involves as a necessary sequence the reversal of the reaction involved in stimulation. It is possible that anesthetics sometimes act by causing a mild stimulation followed by an immediate reversal. The stimulation may be completely hidden so that the observer sees only the reversal, and there is apparently only anesthesia. The fact that in magnesium solutions granule breakdown, which may be regarded as a primary part of the stimulatory gelation, may be inhibited, opens the possibility of explaining the action of magnesium salts in terms of the theories outlined above. It is at least clear that under certain conditions, magnesium may be regarded as tending to inhibit the s.p.r.

However, this is not the only way in which the anesthetic action of magnesium may be interpreted in terms of the s.p.r. It has already been noted that both calcium and magnesium can initiate the s.p.r.[3] Calcium is effective in extremely low concentration. In experiments cited above, the s.p.r. was found to occur in solutions in which one part of isotonic calcium chloride solution was mixed with a thousand parts of isotonic sodium chloride solution. Actually, one part of calcium solution in two thousand parts of sodium solution is sufficient. Not so with magnesium; it must be present in much higher concentration. Experiments were performed in which eggs were placed in various mixtures of isotonic sodium chloride (0.53 M) and isotonic magnesium chloride (0.3 M). In such experiments it is essential to wash the eggs in one dish containing a given mixture and then transfer them to a second. In each transfer a single drop of egg suspension was carried over to 20 cc. of the solution. This procedure is necessary in order to wash the eggs relatively free from calcium ion. Apparently there is some variation between different lots of eggs, nor is it always easy to state precisely just when an s.p.r. occurs and when it is absent, for there is, of course, a gradual transition between a complete reaction and none at all. It is certain, however, that 1 part of magnesium solution to 39 parts of sodium solution is not sufficient to cause an s.p.r. when the eggs are crushed in this mixture. Neither is 1 part magnesium solution to 19 parts sodium solution. In mixtures of 1 part magnesium solution to 9 parts sodium solution, the reaction may or may not occur. This is a boundary concentration and mixtures which contain appreciably more than 1 part of isotonic magnesium solution in 10 parts of the total solu-

[3] It is possible that only calcium is directly involved in the reaction and that magnesium acts by releasing calcium from some chemical union in the protoplasm.

tion are certain to give the reaction. Thus, the magnesium ion is relatively weak in comparison with the calcium ion; the latter is at least a hundred times more powerful in promoting the s.p.r.

These observations suggest a second type of explanation for the anesthetic action of the magnesium ion. It is reasonable to assume that in living systems the magnesium ion can replace the calcium ion. It has been assumed (Heilbrunn, 1928; Heilbrunn and Daugherty, 1933) that the stimulating gelation within cells is due to a release of calcium ion. Suppose now that a cell anesthetized with magnesium has its calcium compounds to some extent replaced with magnesium. If the replacement were complete, stimulation would, according to our theory, require the release of at least 100 times as much free ion if this were magnesium instead of calcium. Thus the magnesium-treated cell becomes relatively resistant to stimulation.

General Discussion

When a living cell is aroused to activity, changes in its protoplasm must occur. These changes are almost certainly not confined to the osmotic membrane of the cell, and the main mass of the living substance is doubtless affected. Protoplasm is a colloidal material and we are therefore faced with a problem in colloid chemistry.

In interpreting stimulation and the action of anesthetics which prevent it, many authors have reasoned from the known effects of stimulating agents and anesthetics on inanimate colloids. The primary information concerning the colloid chemistry of protoplasm has clearly shown the inadequacy of such reasoning. Protoplasm is vastly different from any known inanimate colloid. It is, therefore, essential to study the effects of stimulants and anesthetics on protoplasm itself. Unfortunately, many of the cells which give the most interesting responses when stimulated can not readily be studied from a colloid chemical standpoint. If we are to make progress in this field, the only sure method at our disposal at the present time is to study the effects of stimulants and anesthetics on those cells whose protoplasm lends itself to colloid chemical study. Thus we can determine the effect of ether on various types of protoplasm, and the knowledge so gained is of more importance to the theory of anesthesia than any information derived from non-living materials.

All protoplasm is very sensitive to physical treatment and chemical reagents. It is easily injured, and any excessive stimulation leads to injury or death. Indeed, it is probable that stimulation is equivalent to slight injury. Almost all agents which stimulate or injure protoplasm cause a peculiar reaction in it. The fluid protoplasm becomes stiffer

and this gelation when carried to the point of injury is seen to involve a characteristic vacuolization reaction. Ultraviolet radiation, X-rays, the electric current, sudden pressure, heat, all produce this reaction. How shall we interpret it?

In earlier studies, I have shown that the vacuolization reaction is the same reaction as that which occurs when a cell is torn or broken, that is to say, it is essentially an s.p.r. In many cells the s.p.r. is very easy to study, and one can readily determine the effect of various reagents upon it. But it should be clearly understood that the s.p.r. is an extraordinarily complex reaction or series of reactions. In many respects it resembles blood coagulation. Probably the fundamental reaction of living cells, their power to clot when torn or broken, has been taken over by the blood of many animals, so that there is a deep underlying relationship. It may be remembered that in blood clotting, cell or tissue extracts play important rôles.

In spite of the numerous studies of blood clotting, the process is still very incompletely understood. Only very few authors have studied the clotting of the protoplasmic fluid, so that our knowledge in this field is only at the beginning.

If the attempt is made to develop a logical theory of stimulation based on our present knowledge of the colloidal chemistry of protoplasm, it should be possible in such a theory to account for the action of anesthetics. Apparently protoplasm is in a delicate state of balance between the forces which favor clotting or gelation and those which inhibit or prevent it. With minor reservations, it may be said that stimulation implies a gelation of the main mass of the protoplasm, and anesthesia a solation. If the gelation produced by stimulating agents is essentially an s.p.r., then it should be possible to show that anesthetics prevent or retard the s.p.r. That has been the purpose of the present contribution. For the first time, I have been able to show that fat-solvent anesthetics do actually prevent the s.p.r. in certain types of living material. Moreover, the relation of the magnesium ion to the s.p.r. indicates two possible ways in which this anesthetic ion may retard or prevent the reaction. It has thus been possible to add an additional step in the colloid chemical interpretation of stimulation and anesthesia.

Summary

1. In the presence of low concentrations of calcium, ether inhibits the surface precipitation reaction in *Arbacia* egg cells.

2. Ether and other fat solvents also inhibit the surface precipitation reaction in *Stentor* protoplasm.

3. The magnesium ion can cause a surface precipitation reaction in *Arbacia* eggs, but it is far less potent than calcium. The course of the reaction in magnesium solutions is described.

4. There are two ways in which the presence of magnesium might act to prevent the surface precipitation reaction.

5. The relation of these facts to the colloid chemical interpretation of anesthesia is discussed.

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H-ION CONCENTRATION AS A FACTOR IN THE TOXICITY OF AMINES FOR AMŒBA PROTEUS

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INTRODUCTION

Although it is generally admitted that changes in the pH values of solutions of weak bases influence their penetration rate, the question as to whether free bases or their dissociated ions pass through cell membranes has been a matter of controversy since the time of Overton (1900). In 1921, Crane found that the magnitude of the toxic effect of alkaloids on *Paramecium* varies directly with changes in H-ion concentration, and on comparing the dissociation constants of the bases used, recognized a direct relation between them and the results obtained. She concluded that the undissociated base is responsible for the toxic action. Irwin's investigations (1926, 1930–1) on the penetration of dyes into *Nitella* have added support to this view. Moreover, the facts which many experimental methods have yielded in regard to the nature of protoplasmic boundaries, led Osterhout, in 1931, to state that "electrolytes must travel chiefly in molecular form in passing through this layer."

On the other hand, Brooks, although she found that certain basic dyes (1929) penetrate more rapidly from an alkaline medium, and acid dyes (1932) more rapidly from media of lower pH, was unable to fit the curve in which rate of penetration was plotted against pH to the dissociation curve of the dye. She therefore expresses the guarded opinion that more than one factor is important in determining penetration rate. In view of these facts, we have thought that an examination of the influence of pH changes on the penetration of an extended series of the simplest nitrogen bases, the amines, would be of interest, especially as a very considerable proportion of the physiologically active compounds belong to this class of substance.

Materials and Methods

The amines used were the " Eastman " grade, marketed by the Eastman Kodak Company. The solutions were made up with water twice glass-distilled, from a stock kept in alkali-free glass containers.

Amœbæ, all of the same species, *Amœba proteus,* were found to be satisfactory organisms for the toxicity tests. For observing and counting them a binocular microscope equipped with 10 × oculars and 3.4 × objectives was used in conjunction with the lighting unit described by Chambers and Pollack (1927), and found by them to be satisfactory for the observation of intracellular color changes. The animals were handled by means of a mouth pipette drawn out into a long capillary with a bore sufficiently wide to prevent injury to the plasma-membranes of the cells, and a volume small enough to insure the transfer of but a minimal amount of fluid. Amœbæ for each experiment were picked up individually from a single culture, washed in a large quantity of glass-distilled water, and left over night in this medium. At least 550 amœbæ, selected from those actively crawling and apparently in excellent physiological condition, were subjected to the first washing. This number was reduced by selection to 500 during the transfer to the second (overnight) washing fluid, and again to 450 in seeding the immersion dishes the next morning.

The number of animals used in a given experiment was checked by counting them as they were picked up in the pipette and again after they had been introduced into the immersion dishes containing the amine solutions. The dishes were of the Syracuse type, the under surfaces of which were scored off into quadrants to facilitate counting. During the period of an experiment the dishes were covered with inverted watch glasses to minimize evaporation and prevent the entrance of dust.

Preliminary trials with 10 to 35 amœbæ showed that 30 in a single dish could be quickly and accurately counted without confusion. By a curious coincidence this is the number of animals which Trevan and Boock (1927) consider the mathematically optimum number for the determination of average lethal doses.

With each amine, trial determinations were made to ascertain approximately what concentrations would give definite toxicity effects within a twelve-hour period. The limitation of the experiments to this time interval insured a minimum of bacterial or mould infection and of evaporation, and at the same time gave results fully as satisfactory as those extending over a period of two or three days.

During the experiments the laboratory held a surprisingly constant temperature in the neighbourhood of 20° C.

Four dilutions of the buffered amine were employed at each pH, and the degrees of pH chosen were 6.4, 7.0, and 7.8, which cover the viability range of *A. proteus* without approaching the acid and alkaline death limits too closely. The pH of each stock solution was tested colorimetrically before the introduction of the amœbæ, and, at the end of each experiment various dishes, chosen at random, were examined to make certain that the buffer [1] had kept the cultures at a constant pH.

Criteria of Death

Since the data compiled on the toxicities of the amines are based entirely on mortality counts of amœbæ immersed in solutions of these substances, the necessity of establishing the death point of the amœbæ with the utmost possible degree of accuracy cannot be overestimated. Those criteria must, furthermore, be so definite as to assure their instant recognition, and thus insure identical counts by different observers. Many criteria which on *a priori* grounds might be expected to be of value in determining a death point were debarred for various reasons. For example, death of amœbæ, in the concentrations of amines used, was not accompanied by disintegration or dispersion of the organisms. They remained over a period of many hours as soft coagula. Though cessation of locomotion was quite uniformly a precursor of death, this in itself could not be used as a criterion, for motionless animals transferred to culture media often recuperated. The presence or absence of cyclosis in the internal protoplasm could not be accurately observed with the low power magnifications essential to rapid counting. Rounding of the cell, even over proportionately long periods of time, was found to be unreliable as an indication of death, inasmuch as rounded amœbæ, transferred to a normal culture medium, frequently recovered completely.

In animals immersed in amine solutions, however, there occurred a change in color from the normal gray appearance to a cloudy brown or tan, and such animals never formed pseudopodia, never showed cyclosis when examined under highest power, and never recovered on transfer to culture medium. This contrast between gray and cloudy brown animals could be detected under the low power binocular with ease and accuracy.[2] Color change in the animals was usually preceded by cessa-

[1] Sodium phosphate buffers (M/280) were used, the formulæ for which are given by Cohen and Clark in their Studies on Oxidation Reduction, Hygienic Laboratory Bulletin No. 151, 1928.

[2] In normal animals examined under high powers of the compound microscope there is a sharp contrast of gray crystals and granules against clear bluish-white cytoplasmic matrix. In brown or "bleached" animals, the brown color is not only lodged in the crystals and granules, but the cytoplasmic matrix is cloudy, more opaque and tan in color. This is doubtless due to a protein precipitation at death.

tion of locomotion and by contraction or rounding. In dimethyl and trimethyl amines an initial strong contraction of the animal, accompanied by lifting of the pellicle into numerous petaloid blisters, was followed by swelling of the organism to a spheroid form, after which the color change followed gradually.

As a result of these observations, that point when the color had changed was taken arbitrarily as the death point.

Computation of Toxicity

In any large number of organisms subjected to the action of a toxic substance, a few individuals with low resistance will die very soon, and a corresponding number will survive an equally extended period after the death of the majority, *i.e.*, there will be a distribution of resistance similar to that found in the usual Quetelet population curve. It therefore seemed best, for purposes of comparing toxicities, to disregard the animals falling in the two extreme classes, and to consider the behavior of the middle 50 per cent only. As the total number in any one dish was 30, we therefore considered only that experimental period in which the count of living organisms was from 23 to 7. Furthermore, to express this time as a simple number for purposes of calculation, the midpoint was computed by plotting numbers against time, and observing at what hour the curve crossed a straight line drawn through the number 15. For example, in an experiment for which the results were as follows (methylamine hydrochloride M/100 and M/200):

	Amoebae alive at end of					
	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
M/100.....	29	21	6	1	0	
M/200.....	30	30	25	16	4	1

the midpoint for the M/100 series averages 4¾ hours, and for the M/200 series, 8¼ hours. A time interval in which there occurs a sharp reduction in numbers has also been designated as the period of "critical break" in the population curve. More precisely, the mortality times for the example given would be 4¾ hours and 8¼ hours respectively for the M/100 and M/200 solutions, and that interval between 4 and 6 hours in the M/100 solution would be designated as an interval of "critical break."

Experiments and Results

Toxicity of Primary Amines

Methylamine hydrochloride.—Trial determinations made with this amine suggested the use of concentrations from M/100–M/800 at each pH. A striking contrast was observed between the gradual decrease in number of living animals in concentrations of the amine buffered to pH 6.4, and the rapid death rate in the same concentrations buffered to pH 7.8. Remarkable uniformity in the behavior of the amine was observed. With one culture,[3] for example, in concentrations of M/100 to M/800

Protocol I

Methylamine hydrochloride, M/100–M/800

	Conc. of amine	Imm. time	No. amœbæ	2 hrs.	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.
At pH 6.4	Control	8:25	30	30	29	29	29	29	29
	M/100	8:27	30	29	27	21	21	15	11
	M/200	8:30	30	29	25	25	25	21	14
	M/400	8:32	30	27	25	25	25	22	21
	M/800	8:35	30	30	30	29	29	27	26
At pH 7.0	Control	8:38	30	30	30	30	30	30	30
	M/100	8:40	30	29	21	6	1	0	
	M/200	8:42	30	30	30	25	16	4	1
	M/400	8:45	30	28	27	27	24	23	23
	M/800	8:47	30	29	29	28	26	25	22
At pH 7.8	Control	8:50	30	30	30	30	30	29	29
	M/100	8:53	30	28	8	2	2	0	
	M/200	8:55	30	19	6	1	0		
	M/400	8:58	30	22	10	6	0		
	M/800	9:00	30	23	15	8	6	3	3

at pH 6.4, the total count never fell to 7 over the entire 12-hour period. In a concentration of M/100 at 7.0, a critical break occurred in the count of survivors between 4 and 5 hours; in a concentration of M/200, a more gradual reduction of numbers began between 4 and 5 hours and continued for 4 hours; but in concentrations of M/400 and M/800, the total count never fell to 7. At a pH of 7.8, in concentrations of M/100 and

[3] For each experiment, all the animals were taken from one culture or "race." No substitutions were made from one record to another, for although the mortality-time in any one day's record shows a consistently more rapid mortality rate in the amine buffered to 7.8 than in the concentrations buffered to 6.4, the viability of the amœbæ of different cultures varies appreciably. The interchanging of records would, therefore, tend to introduce a new source of error.

M/200, the critical break in the survivor curve occurred between 2 and 3 hours; in M/400, between 3 and 4 hours, and in M/800, between 4 and 5 hours. (See Protocol I.)

Failure to reduce the number of amœbæ in the acid group to the lower limit arbitrarily set for mortality counts, *i.e.*, 7, called for repetitions of this series in higher concentrations. Experiments were therefore carried out in which M/10–M/80 concentrations of methylamine buffered to pH 6.4 were used.

Ethylamine hydrochloride.—Ethylamine hydrochloride was used in concentrations of M/100, M/200, M/400, and M/800. A typical protocol shows, as in the case of methylamine, a definite contrast between the toxicity of concentrations buffered to pH 6.4 and the same concentrations buffered to pH 7.8. The contrast between these extremes is, however, not as sharply defined as that found in the methylamine series at similar concentrations, since the arbitrary number, 7, is reached in the acid series in both concentrations of M/100 and of M/200. In the pH 6.4 series also sharp breaks occur in the two higher concentrations at a period of approximately 5 hours, though in the lower concentrations, M/400 and M/800, the number of amœbæ is not reduced to 7 during the entire 12-hour period. In the concentrations buffered to pH 7.0, sharp breaks occur at about 3 hours in the two stronger concentrations and in about 5 hours in M/400. In the weakest concentration employed the number is not reduced to 7 in the 12-hour period. At a pH of 7.8, the critical break came between 2¾ and 3¾ hours in the three strongest concentrations, and at approximately 5¾ hours in the weakest.

Propylamine hydrochloride.—Propylamine hydrochloride was used in concentrations of M/100, M/200, M/400 and M/800. A typical protocol for this amine shows that although the contrast in toxicity between concentrations buffered to pH 6.4 and to pH 7.8 is not as conspicuously evident as in the case of both methyl and ethyl amines, it is, nevertheless, readily observable. Except in the case of M/100 propylamine hydrochloride of the acid and the neutral series, the critical breaks in the count of survivals are limited to the alkaline series. In this series the mortality time for the two stronger concentrations ranges between 5 and 6¾ hours, while the death rate in the M/800 concentration is gradual, and does not reach the lower limit of the mortality count within 12 hours.

Butylamine hydrochloride.—This amine was used in concentrations of M/100, M/200, M/400 and M/800. In a typical protocol, the difference in toxicities of the acid, neutral, and alkaline series is definite. In the pH 6.4 series no critical break in resistance is shown except in the strongest concentration, M/100. The neutral series shows critical

breaks in the two stronger concentrations, and a uniform gradual decrease in numbers in the other two concentrations after a period of 8–10 hours. However, in the alkaline series sharp breaks appear at about 3 to 3¾ hours in M/100, M/200; at about 6 hours in M/400 concentrations; and a rapid decrease is shown in a concentration of M/800 though the count is not reduced to 7.

Relative Toxicities of Primary, Secondary and Tertiary Amines

Primary amines.—In the previous section, no comparison was made of the relative toxicities of the four primary amines. Since each day's protocol concerned itself with the history of but 30 amœbæ, and since within so small a population the true mortality curve may not be apparent, the data from a large number of experiments were combined in order to reduce the chances of error. From typical groups of 90 to 180 amœbæ the averages given in Table I were derived.

TABLE I

Mortality-time at a pH of 7.8 (limit 12 hours)

	M/100	M/200	M/400	M/800
	hours	*hours*	*hours*	*hours*
Methylamine.....	2.91	3.00	3.16	4.16
Ethylamine.....	2.96	3.30	3.42	5.96
Butylamine.....	3.75	4.95	6.25	(Non-toxic in 12 hrs.)
Propylamine.....	5.10	5.86	6.75	(Non-toxic in 12 hrs.)

If the average mortality-time for any given concentration at a pH of 7.8 is taken as an indicator of toxicity, the degree of toxicity of the four primary amines may be expressed as follows: methylamine> ethylamine> butylamine> propylamine. The order of these amines on the basis of degree of toxicity does not, therefore, coincide with their order in the chemical series.

Secondary and tertiary amines.—A series of trial experiments made with dimethyl, diethyl, and trimethyl amine hydrochlorides in the concentrations used for the primary series showed at once that such concentrations were far too non-toxic to give comparable mortality-time records within a period of 12 hours. The strength of the concentrations of the secondary amines was increased to M/10, M/20, M/40 and M/80, and of the tertiary amine to M/2, M/4, M/8 and M/16. The use of such concentrations necessarily introduces new problems of degree of dissociation and osmotic pressure and the results obtained are difficult to interpret satisfactorily. In dimethylamine a certain degree of cor-

relation with the behavior of the primary amines is observed in that the alkaline series (pH 7.8) shows the greatest degree of toxicity; critical reductions in the total count appearing in the three strongest concentrations between 2 and 4 hours, and in M/80 between 4 and 6 hours. The shrunken appearance of the amœbæ in the M/10 concentrations of the secondary amines supports the conclusion that toxicity counts of animals immersed in solutions of such high osmotic pressure are of questionable value. The abrupt fall in numbers in the trimethylamine series in concentrations of M/2 and M/4 is probably attributable to the same factor. The mortality rates in the M/8 and M/16 concentrations show a fair degree of uniformity.

Discussion

Without exception, the experimental data on the toxicities of the primary amines lend further support to the view that weak bases penetrate more rapidly from alkaline than from acid solutions. The possibility must be considered that the increased toxicity is an expression of some direct action by the more basic media on the cells themselves. This seems unlikely since it was noted that the viability of the amœbæ was approximately equal in the control solutions at all of the H-ion concentrations used. There remains, then, the consideration of whether the changes in degree of dissociation of the amines are quantitatively related to the degree of toxicity produced.

In a M/30 solution of methylamine hydrochloride at a pH of 6.4, the "lethal time" for one culture of amœbæ was 4.25 hours. The amount of free base present (using the value 5×10^{-4} for the dissociation constant) is found to be 1.65×10^{-6}. Equivalent amounts of free base are present in a M/121 solution at a pH of 7.0, and in a M/763 solution at a pH of 7.8. Experimental data give (for the same culture) the same lethal time at a pH of 7.0 in a M/100 concentration, and, at a pH of 7.8, in a M/800 concentration. The parallel between the increase in amount of undissociated base and the decrease in length of the toxicity time is striking.

In two series of ethylamine hydrochloride immersions the lethal time, at a pH of 6.4, in a M/100 solution was 5.75 hours, and in a third series, 6.5 hours. The concentration of free base present (using the value 5.6×10^{-4} for the dissociation constant of the amine) is 0.44×10^{-6}. At a pH of 7.0, an equivalent amount is present in a M/407 solution, and at a pH of 7.8, in a M/2539 solution. Experimental data show, even in the presence of a great viability difference in the three races (cultures) used, very consistent results. At a pH of 7.0, in the first two series, the lethal time 5.75 hours was reached in the M/400

solutions, and in the third series the lethal time 6.5 hours is found in this same concentration. However, at a pH of 7.8, though the concentration toxic in 6 hours is M/800 for all three series, this is in wide disagreement with the expected figure, *i.e.*, M/2539.

In the propylamine hydrochloride series the divergence between calculated and actual concentrations is even more pronounced. Agreement in behavior of resistant and weak races (cultures) is, however, again seen to be strikingly consistent. In two races with lethal times of 7 hours and 6.5 hours respectively in M/100 solutions at a pH of 6.4, the same lethal periods were found at pH 7.0 in M/150 solutions, and at pH 7.8, in M/400 solutions. Calculations made on the basis of 4.7×10^{-4} as the dissociation constant, gave the expected figures as follows: for a pH of 7.0, M/401; for a pH of 7.8, M/2539. This discrepancy in the case of propylamine clearly indicates the activity of some important and unknown factor.

No values for the dissociation constant of butylamine can be found in the literature.

Under the conditions of these experiments it appears that although a close relationship exists between the amount of undissociated base present and the degree of toxicity of methyl and ethylamines, this agreement does not hold true for propylamine.

Conclusions

1. Under the conditions of the experiment, the degree of toxicity of solutions of the hydrochlorides of methyl, ethyl, propyl and butyl amines varies uniformly with the H-ion concentration as follows: the toxicity at a pH of 7.8> at a pH of 7.0> at a pH of 6.4.

2. In solutions of methylamine hydrochloride, the parallel between the increase in amount of undissociated base and the decrease in length of toxicity time is striking. In ethylamine solutions, this relation holds in concentrations buffered to a pH of 6.4 and 7.0, but not for the solutions buffered to a pH of 7.8. In the case of propylamine, though all the experimental data are convincingly uniform, the divergence between calculated and actual toxicity concentrations is even more pronounced. The presence, therefore, of some other important factor or factors is clearly indicated.

3. The relative degree of toxicity of the first four primary amines may be expressed as follows: methylamine> ethylamine> butylamine> propylamine.

4. Solutions of the hydrochlorides of dimethyl and diethyl amines were found to be less toxic than methyl and ethyl amines.

5. In the case of diethylamine, there is a certain degree of correspondence with the behavior of primary ethylamine, in that the alkaline series (at a pH of 7.8) shows the greatest degree of toxicity.

6. Solutions of triethylamine are still less toxic than those of the secondary ethylamine. To obtain mortality-time records within twelve hours, the effective concentration of tertiary ethylamine reaches a point where the high osmotic pressure causes shrinkage of the cells.

We wish to express our thanks to Dr. Kenneth C. Blanchard, of Washington Square College, for his valuable criticism, and to Dr. Samuel E. Hill, of Rockefeller Institute, for his interest in checking our calculations.

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STUDIES ON THE CILIATES FROM FRESH WATER MUSSELS

II. The Nuclei of Conchophthirius Anodontæ Stein, C. Curtus Engl., and C. Magna Kidder, during Binary Fission

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In a previous paper (Kidder, 1934) I have given a description of the general morphology, neuromotor apparatus, and vegetative nuclei of the three species of *Conchophthirius* to be discussed in the following pages. Mention was made of the occurrence of binary fission, and the general appearance of the metaphase spindles was figured. It is the purpose of the present paper to report the cytological details of the nuclear phenomena during division and to point out differences from and similarities to the nuclei of related forms.

Relatively few of the many descriptions of ciliate division give a detailed account of the number and behavior, during binary fission, of the micronuclear chromosomes. Especially noticeable is the general lack of close observations as to the period between the metaphase and the anaphase stages with regard to the method of chromosomal division. This situation is quite obviously due to the difficulty of observation of so small a cell element and also in part to the usually short duration of this stage. Whether ciliate chromosomes divide transversely or longitudinally is of great theoretical significance, as was clearly pointed out by Calkins (1930*a*). A great many more observations on this point are needed before we can formulate any general conclusions.

Due to the large size of the micronucleus of *Conchophthirius anodontæ*, the great abundance of material, and the ease of fixing and staining, I have been able to make a detailed study of the chromosomal division in this species. Although the other two species studied, *C. curtus* and *C. magna*, were very plentiful, their micronuclei are so small, as pointed out before (Kidder, 1934), that I was unable to observe the chromosomes clearly and so could not be sure of the exact method of chromosomal division. In all three species the changes occurring in the macronuclei during fission could be followed with ease.

The material used in this study was obtained from the same sources as those previously reported (Kidder, 1934). The preparations were

made at the Marine Biological Laboratory at Woods Hole, Massachusetts.

Technique

The method of obtaining the ciliates for study of the division process was similar to that used in the study of *Conchophthirius mytili* (Kidder, 1933). As in that species, the present ciliates can be selected for fixation by observing, through a dissecting binocular, the condition of the macronucleus. In this manner practically any stage can be obtained in a very short time. However, unlike *Conchophthirius mytili*, the present ciliates are so numerous that a single mussel will often yield all of the various stages of binary fission.

The fixatives employed were Flemming's, Schaudinn's, Bouin's, and Gilson-Carnoy's fluids and sublimate-acetic in 95 per cent alcohol. Flemming's fluid was followed by bleaching in H_2O_2.

The stains giving uniformly good results were Heidenhain's and Delafield's hæmatoxylins, the Borrel stain and the Feulgen thymonucleic acid reaction. By far the best preparations were obtained by the Feulgen reaction following Schaudinn's fluid or sublimate-acetic in 95 per cent alcohol, and Heidenhain's hæmatoxylin (long method) after Schaudinn's or Flemming's fluids.

I employed a modification of the picric acid destaining method of Tuan (1930) following Heidenhain's hæmatoxylin. For this modification I am indebted to Messrs. T. T. Chen and R. Wichterman. After staining, the coverglasses are rinsed in water and placed in a saturated aqueous solution of picric acid. Usually twenty to thirty minutes are required to extract a sufficient amount of stain. Critical examination can be made at any time during the destaining. By passing the coverslip over the mouth of a bottle containing concentrated ammonium hydroxide the brownish appearance imparted by the picric acid is removed. The fumes change the brown of the picric to a bluish black and render the organism quite clear. Care should be taken not to expose the coverslips to the ammonium hydroxide for a longer period than is needed to produce the bluish color, as excess will result in the swelling of the organism. When sufficient stain has been extracted, so that the cytoplasm is a pale gray, the coverglasses are washed in running water for thirty minutes, dehydrated and mounted.

Observations

Conchophthirius anodontæ Stein

Micronucleus.—The first sign of division in this ciliate is to be found in the swelling and loss of staining capacity of the micronucleus.

From the compact vegetative condition the chromatin becomes flocculent and the nuclear membrane moves away from the central mass leaving a clear area. This clear area is no doubt partly the result of shrinkage brought about by fixation. The micronucleus moves out of its pocket in the macronucleus and takes up a position in the mid-region of the cell. The chromatin becomes irregularly disposed in a reticulate fashion (Fig. 1, *A*). Swelling continues and the chromatin condenses into a twisted band, which at first is quite irregular. This is similar to a spireme stage. (Fig. 1, *B*). I am unable to say whether the spireme band is continuous or broken at this time. In the clear space at the poles of the chromatin mass delicate spindle fibers can be seen. These fibers appear to be pushing out from the central matrix. The spireme band becomes more basophilic and breaks into many small segments. These segments orient themselves along the long axis of the nucleus, with their ends pointing toward the rapidly forming spindle fibers (Fig. 1, *C*). The whole nucleus is undergoing elongation. The chromatin making up the bands is disposed in irregularly spherical chromomeres of considerable size. In carefully differentiated material it is possible to count these bands even at this early stage. The number is twelve. The metaphase plate is formed by a condensation of the twelve bands into definite but rough chromosomes, a concomitant elongation of the nucleus, and completion of the division spindle (Fig. 1, *D*). The chromosomes of the metaphase plate are always lined up along the long axis of the spindle.

I have never seen any evidence of an endosome at any stage in the micronuclear mitosis, such as was found by Wenrich (1926), Manwell (1928) and Turner (1930).

About this time the first indication of actual chromosomal division may be seen. A longitudinal split occurs in each chromosome and eventually the two daughter chromosomes are clearly discernible (Fig. 1, *E*). Material stained after the Feulgen reaction was the most satisfactory for this stage as the chromosomes are sharply outlined and there is none of the haziness so often seen in material stained in hæmatoxylin. Unlike the majority of ciliates, the metaphase appears to be of rather long duration and dozens of well-prepared individuals were obtained for study. In no case, however, was I able to detect the split in all the chromosomes at the same time. The metaphase plate is quite regular and all the chromosomes are of approximately equal length. The migration of the daughter chromosomes to their respective poles is again a relatively slow process, judging from the great number of anaphases obtained. The daughter halves slip by one another resulting in a broad band of chromosomes in the central portion of the spindle. Each daughter increases somewhat in size. It is possible at all times during

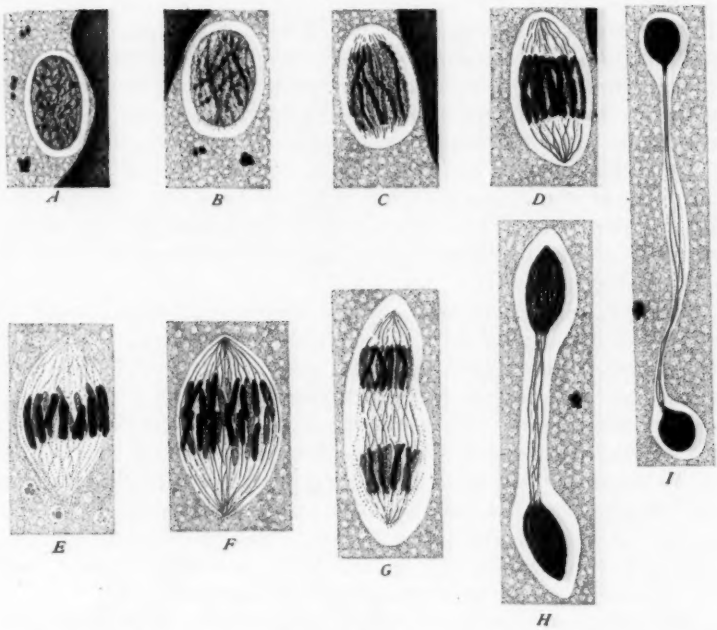

FIG. 1. The micronucleus of *Conchophthirius anodontæ* during mitosis. All except *E* were fixed in Schaudinn's fluid, stained with Heidenhain's hæmatoxylin and destained in aqueous picric acid. *E* was fixed in sublimate acetic in 95 per cent alcohol and stained after the Feulgen reaction. Drawings were made with the aid of a Promar projector. × 2644.

A. Early prophase with the chromatin in the flocculent state.

B. Spireme stage. The spindle fibers are just beginning to appear.

C. Late prophase. Twelve long strands are oriented toward the poles of the forming spindle.

D. Early metaphase. Twelve chromosomes.

E. Metaphase. Twelve fully contracted chromosomes. Some show definite longitudinal splits while in others only the notched condition of the ends indicates where the split will appear.

F. Early anaphase. Daughter chromosomes migrating towards their respective poles.

G. Late anaphase. The two groups of twelve chromosomes each are well separated.

H. Early telophase.

I. Late telophase. Separation spindle clearly shown.

this slipping-past process to count the twenty-four daughter halves (Fig. 1, *F*). Not only is it possible to make consistent counts but because of their proximity it is usually possible to detect which two halves were originally a single chromosome. The metaphase split and the early anaphase migration are undoubtedly the most interesting features of this extremely large and clear micronucleus. I have never seen any indication of the dumb-bell-shaped chromosomes of the early anaphase that occur so frequently in ciliates, and because of the extreme numbers of all stages of mitosis available for study, the large size, and the clear staining properties of this micronucleus, I feel certain that the activity described above is accurate as far as *Conchophthirius anodontæ* is concerned.

The late anaphase is quite regular. Twelve daughter chromosomes migrate to the poles of the now elongated spindle (Fig. 1, *G*). Here they contract into a typical spear-head mass. The fibers of the spindle retain their form between the two daughter nuclei (Fig. 1, *H*) even to the very late telophase. The formation of the daughter nuclei is regular, the chromatin contracting into two homogeneous spheres (Fig. 1, *I*). As the daughter nuclei move apart a long " separation spindle " is pulled out between them. In the flared portion of the separation spindle a number of fibers persist for some time. The separation spindle and contained fibers gradually fade out and the two micronuclei round up and take up their positions against the daughter macronuclei.

Macronucleus.—During the early prophases of the micronucleus, the macronucleus of *Conchophthirius anodontæ* swells slightly and, as the late prophase of the micronucleus sets in, migrates to a central position in the cell. During the metaphase of the micronucleus, the macronucleus elongates in the direction of the long axis of the cell and internal changes take place. The chromatin becomes evenly granular and as constriction starts an area of more deeply staining chromatin forms in the central region. This island is marked off from the major portion of the macronucleus by a halo of less deeply staining chromatin (Fig. 2, *A*). The differentiation of the central area is not the same in all individuals, for in some the nuclear granules appear evenly distributed until a later stage. The central mass is the residual chromatin characteristic of the Conchophthiriidæ. The residual mass condenses into a densely staining sphere between the dividing daughter halves of the macronucleus (Fig. 2, *B*). The macronuclear membrane flares out to accommodate the residual mass just as it does in *Conchophthirius mytili* (Kidder, 1933*a*) and *Ancistruma isseli* (Kidder, 1933*b*). Subsequent pulling apart of the daughter halves leaves the residual mass near the cell center (Fig. 2, *C*). Finally the connections break and the residual mass rounds up

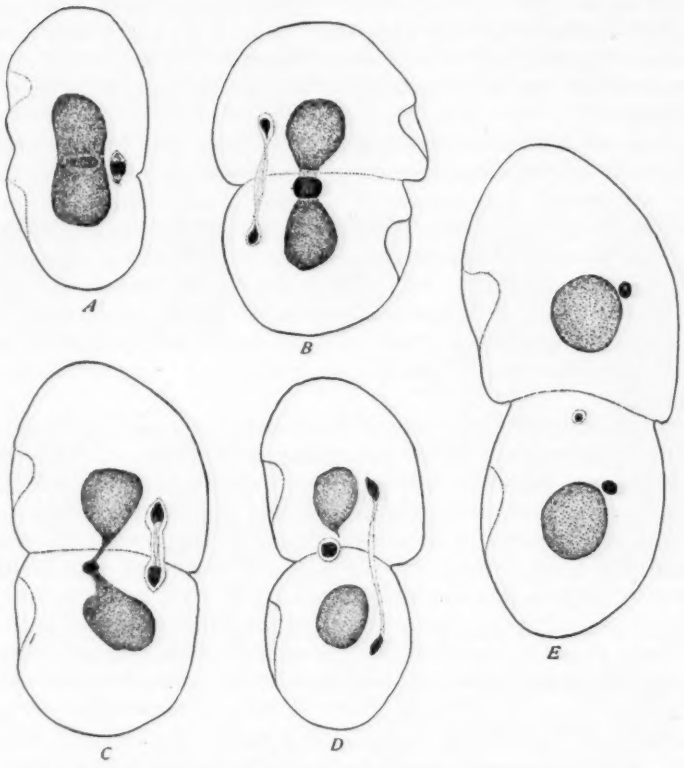

FIG. 2. The macronucleus of *Conchophthirius anodontæ* during binary fission. Camera lucida drawings. × 511.

A. Earliest evidence of the formation of the ball of residual chromatin. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

B. Residual ball somewhat contracted and lying between the separating halves of the macronucleus. Schaudinn's fluid—Heidenhain's hæmatoxylin.

C. Further contraction of the residual ball and separation of the macronuclear halves. Schaudinn's fluid—Heidenhain's hæmatoxylin.

D. Daughter macronuclei have separated. Residual ball has rounded up within a fragment of the old macronuclear membrane. Gilson-Carnoy's fluid—Delafield's hæmatoxylin.

E. Plasmotomy well under way. Residual chromatin is fading out in the cytoplasm. Sublimate acetic—Feulgen reaction.

(Fig. 2, *D*). Plasmotomy proceeds and the residual chromatin, in the cytoplasm of either of the daughter ciliates, starts to disintegrate and rapidly loses its affinity for basic stains (Fig. 2, *E*) until finally it becomes completely absorbed. Plasmotomy may or may not be completed before the entire dissolution of the residual chromatin has occurred.

In a few cases of division in this species no residual chromatin was visible, the macronucleus dividing cleanly. These cases were similar to the reorganized exconjugants of *Conchopthirius mytili* (Kidder, 1933*a*). I believe it highly possible that the cases of cleanly dividing macronuclei of *Conchophthirius anodontæ* also represent organisms shortly after conjugation. My belief is based on the presence of considerable numbers of reorganizing individuals found during the first month of this investigation (June, 1933), indicating that conjugation had previously taken place. I will have more to say on this matter in a later paper.

Irregularities of division, which I am inclined to regard as abnormal and probably pathological, are occasionally encountered. A few dividing organisms showed an obvious upset in the normal procedure. In some cases the macronuclear division was very asymmetrical, the greater portion being included in one daughter organism (Fig. 7, *A*). In others the nuclear division failed to keep pace with plasmotomy, all of the chromatin remaining in one daughter, leaving the other devoid of nuclei (Fig. 7, *B*). In still other cases only a small portion of the macronucleus is passed to one daughter ciliate while the other daughter retains most of the macronuclear material and all of the micronuclear material (Fig. 7, *C*). In some of these cases we have visible evidence of the possibility of the formation, through faulty fission, of an amicronucleate race. Whether or not this ever occurs, or indeed whether or not either daughter is viable, I cannot say.

Conchophthirius curtus Engl.

Micronucleus.—The general activity of the micronucleus of *Conchophthirius curtus* is very similar to that of *C. anodontæ* but because of its small size it is impossible to follow the finer details of exact chromosome number and method of chromosomal division, at least in my material.

The early prophase occurs as the micronucleus emerges from its pocket in the plastic macronucleus. It swells slightly, the chromatin becoming loosened and finely granular and less deeply staining than in the vegetative state (Fig. 3, *A*). The micronucleus now elongates,

always in the plane of the long axis of the body of the ciliate. The chromatin becomes condensed into many granular threads, lined up along the long axis (Fig. 3, *B*). These threads contract, increase in staining capacity, and form the chromosomes of the metaphase plate (Fig. 3, *C*). In the meantime spindle fibers have pushed out to form a rather sharply pointed spindle. The chromosomes lie very close to one another and I have not been able to make accurate counts, even after the Feulgen reaction. The next stage is not clear. It is very hard to determine

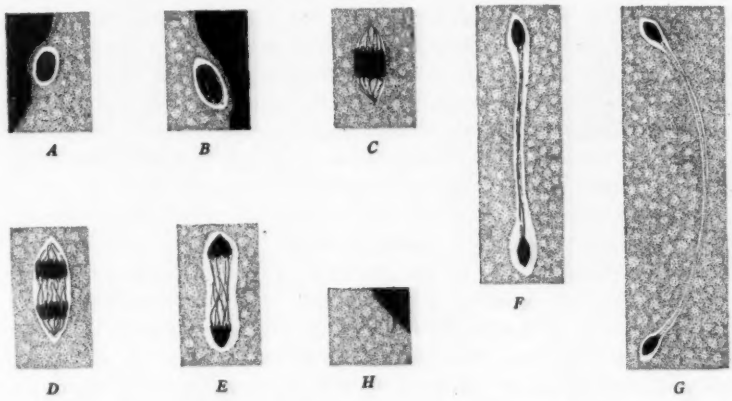

FIG. 3. The micronucleus of *Conchophthirius curtus* during mitosis. Camera lucida drawings. × 2663.

A. Early prophase. Micronucleus migrating from its pocket in the macronucleus. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
B. Prophase. Chromatin in strands. Sublimate acetic—Feulgen reaction.
C. Metaphase. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
D. Anaphase. Schaudinn's fluid—Heidenhain's hæmatoxylin.
E. Early telophase. Schaudinn's fluid—Heidenhain's hæmatoxylin.
F. Telophase. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
G. Later telophase. Schaudinn's fluid—Heidenhain's hæmatoxylin.
H. Reconstruction of one daughter micronucleus. Evidence of separation spindle still seen. Sublimate acetic—Heidenhain's hæmatoxylin.

whether a broad band of chromosomes represents a contraction from the prophase or a migration at early anaphase. Of this I am certain, however, that in the dozens of cases of this stage studied I have never found the dumb-bell-shaped chromosomes such as were found in *Conchophthirius mytili* (see Plate II, Fig. 16 in Kidder, 1933*a*). This would lend support to the view that chromosomal division is longitudinal as in *C. anodontæ*, and that in the early anaphase migration the two daughters slip past one another.

The later anaphase is quite clear. Two groups of evenly distributed chromosomes are seen toward the poles of the spindle (Fig. 3, *D*). Between the two daughter groups of chromosomes the mid-fibers are always quite clear. Further elongation of the micronucleus and further migration of the daughter chromosomes completes the anaphase (Fig. 3, *E*). Very shortly the chromosomes lose their identity in the spearhead telophase chromatin mass (Fig. 3, *F*). These daughter micronuclei pull further and further apart, stretching out an extremely long connecting strand, composed of the mid-fibers and the nuclear membrane (Fig. 3, *G*). It is usually not until the daughter micronuclei have entered the daughter macronuclei that this connection is ruptured. For some time thereafter traces of the connecting strand can be seen extending from the otherwise contracted micronuclei (Fig. 3, *H*).

Macronucleus.—The phenomena accompanying the division of the macronucleus of *Conchophthirius curtus* so closely resembles that of *C. anodontæ* that I shall not dwell at length on this subject.

The furrowed macronucleus migrates into the mid-region of the cell and elongates. A heavily staining ball of chromatin becomes differentiated from the rest of the macronuclear chromatin in the central region. This ball is surrounded by a less densely staining halo (Fig. 4, *A*). As the macronucleus constricts the ball contracts (Fig. 4, *B*) and remains between the daughter macronuclei (Fig. 4, *C*). The residual mass retains its connections with the daughter macronuclei for some time (Fig. 4, *D*). It is finally freed and then rounds up in the cytoplasm of either daughter ciliate (Fig. 4, *E*). The daughter macronuclei lose their smoothly granular condition and again become furrowed and the residual chromatin disintegrates and disappears.

I have found several cases where no residual chromatin was formed, the macronuclei separating cleanly. As in *C. anodontæ* and *C. mytili* I am inclined to regard these cases as ciliates shortly after exconjugant reorganization. I have found numerous conjugating pairs and many reorganizing individuals, and the cases in which a residual mass was lacking were in about the proportions one might expect if these cases do represent exconjugants. Future investigation on the conjugation of these ciliates may shed more light on this interesting problem.

Abnormalities of fission were rarely encountered in this species. Three cases of precocious plasmotomy were observed where the macronucleus and the micronucleus were included in a single daughter, the other daughter being entirely devoid of nuclear material (Fig. 7, *D*).

Conchophthirius magna Kidder

Micronucleus.—As I have previously stated (Kidder, 1934), *Conchophthirius magna* possesses, in about 90 per cent of the cases, two small,

compact vegetative micronuclei. Dividing individuals, in which the micronuclei are quite easily seen, confirmed this percentage. About 10 per cent of the individuals possess but one micronucleus. In two cases,

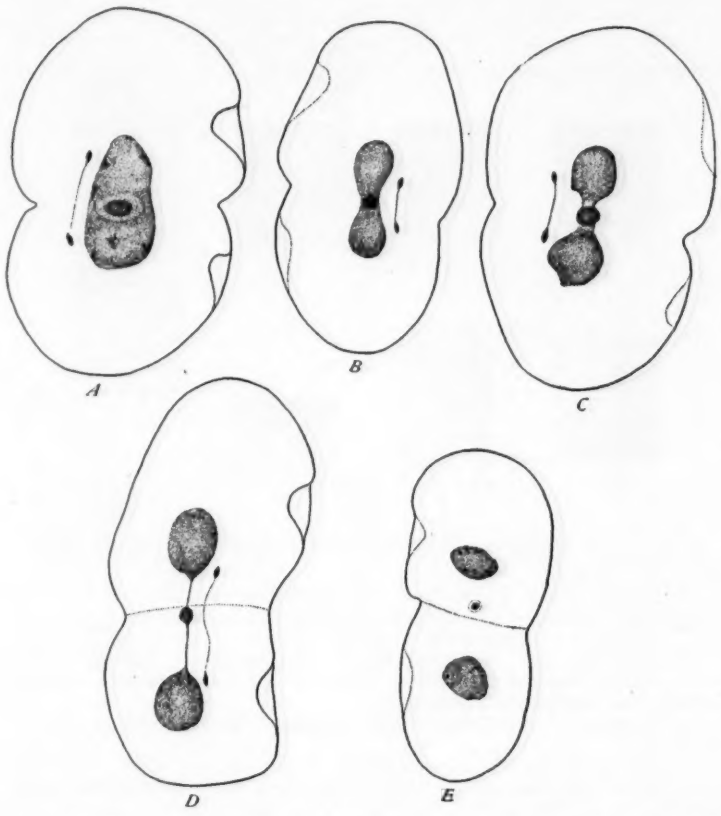

FIG. 4. The macronucleus of *Conchophthirius curtus* during binary fission. Camera lucida drawings. × 521.

A. Formation of the ball of residual chromatin. The macronucleus is still in a furrowed condition. Schaudinn's fluid—Heidenhain's hæmatoxylin.

B. Constriction of macronucleus. Residual chromatin quite compact. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

C. Further constriction of the macronucleus and separation of the residual chromatin. Flemming's fluid—Heidenhain's hæmatoxylin.

D. Residual chromatin connected to daughter macronuclei. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.

E. Daughter macronuclei reorganized. Residual chromatin disintegrating. Sublimate acetic—Feulgen reaction.

however, I was unable to see any micronuclei. These individuals, though in the early stages of fission, contained an enormous amount of food which, I believe, obscured the spindle or spindles that may have been present.

The micronuclei of *C. magna* appear to possess a greater tendency to remain near or in contact with the macronucleus during the prophase, metaphase and anaphase stages than do those of *C. anodontæ* and *C.*

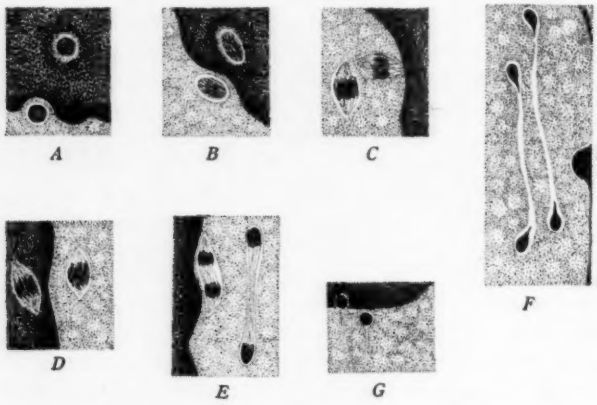

FIG. 5. The micronuclei of *Conchophthirius magna* during mitosis. Camera lucida drawings. × 2076.

A. Early prophase. Gilson-Carnoy's fluid—Feulgen reaction.
B. Late prophase. Sublimate acetic—Heidenhain's hæmatoxylin.
C. Metaphase. Sublimate acetic—Feulgen reaction.
D. Early anaphase. Schaudinn's fluid—Feulgen reaction.
E. One micronucleus in middle anaphase and one in late anaphase. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
F. Telophase. Gilson-Carnoy's fluid—Feulgen reaction (counterstained in Borrel II).
G. Late telophase. The micronuclei are compact and spherical but still connected to the long separation spindles. Schaudinn's fluid—Heidenhain's hæmatoxylin.

curtus. Swelling of the micronuclei results in a finely granular condition at the onset of mitosis (Fig. 5, *A*). The chromatin becomes oriented into granular threads extending nearly the entire length of the somewhat elongated nucleus (Fig. 5, *B*). These threads contract and form rather definite chromosomes on the equatorial plate of the now fully formed spindle (Fig. 5, *C*). These chromosomes are again too small and too compact to enable one to count them. The beginning of the anaphase is again, as in *C. curtus*, a question of interpretation. I believe Fig. 5, *D*

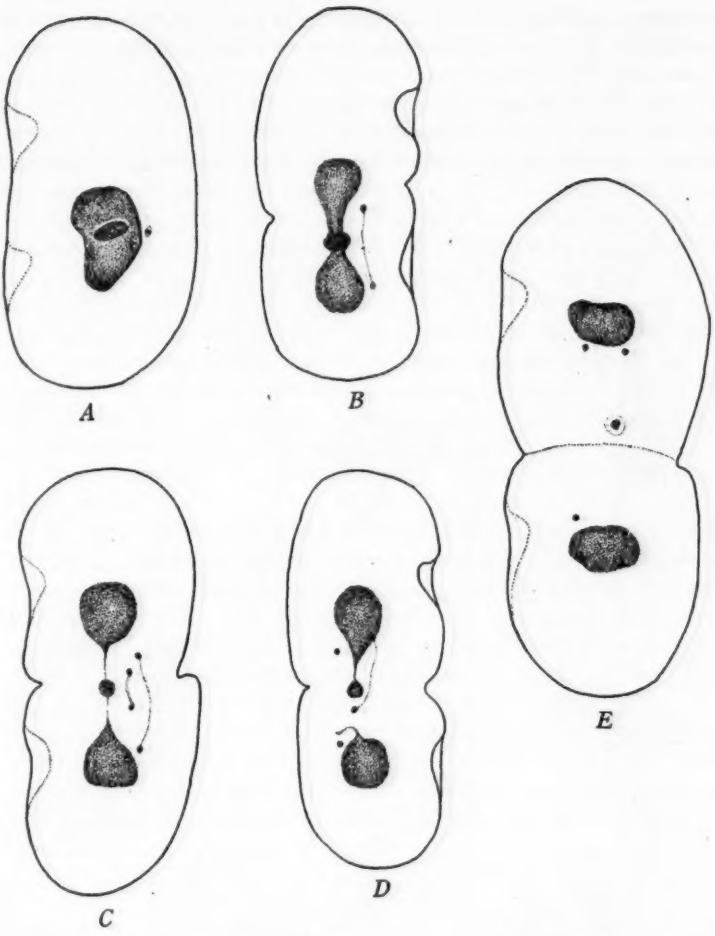

FIG. 6. The macronucleus of *Conchophthirius magna* during binary fission. Individuals were all fixed in sublimate acetic in 95 per cent alcohol and, with the exception of *E*, were stained after the Feulgen reaction. *E* was stained in Heidenhain's hæmatoxylin. Camera lucida drawings. × 350.

A. Formation of ball of residual chromatin.

B. Constriction of macronucleus and separation of residual ball. The individual possessed a single micronucleus.

C. Separation of daughter macronuclei.

D. Separation of one daughter macronucleus from residual ball.

E. Reorganization of daughter macronuclei and disintegration of residual chromatin.

represents the slipping past of daughter chromosomes on their way to their respective poles. I have never seen any dumb-bell-shaped chromosomes that would indicate transverse division.

The later anaphases are quite regular and very similar to those of *C. curtus* (Fig. 5, *E*). The mid-fibers are clearly demonstrated between the two daughter groups of chromosomes. In the telophase the daughter

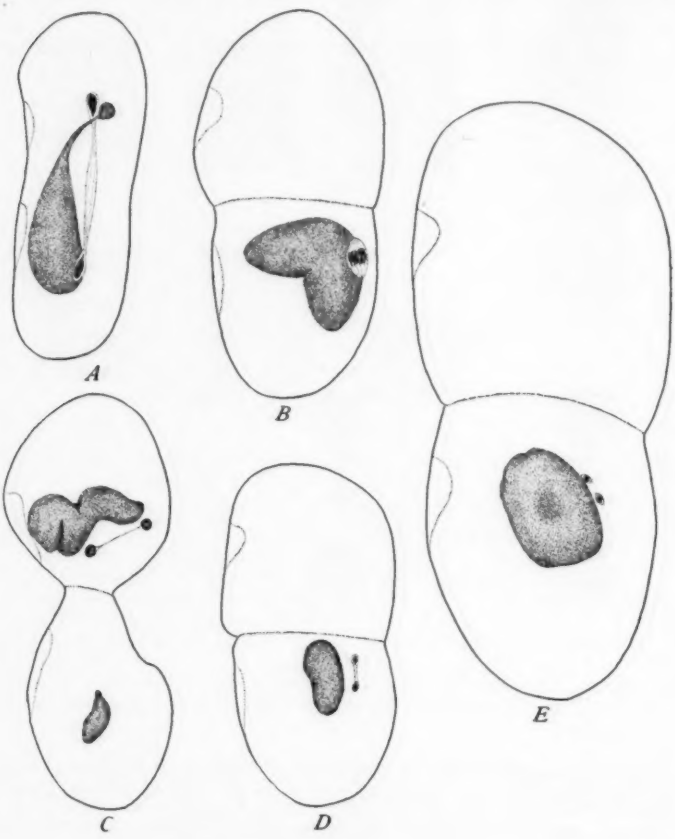

FIG. 7. Abnormal divisions. × 486.

A. Conchophthirius anodontæ. Schaudinn's fluid—Feulgen reaction.
B. C. anodontæ. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
C. C. anodontæ. Gilson-Carnoy's fluid—Heidenhain's hæmatoxylin.
D. C. curtus. Sublimate acetic—Heidenhain's hæmatoxylin.
E. C. magna. Bouin's fluid—Heidenhain's hæmatoxylin.

micronuclei pull out a long connecting strand (Fig. 5, *F*) which, in some cases, persists until the four compact daughter micronuclei enter the macronuclei (Fig. 5, *G*).

Macronucleus.—As the micronuclei prepare for mitosis the macronucleus of *Conchophthirius magna* begins to lose its furrowed condition and finally becomes evenly granular. A large mass of deeply staining chromatin, surrounded by a lightly staining halo, is differentiated near its center (Fig. 6, *A*). This mass contracts into a ball and is left in the division plane as the daughter halves of the macronucleus pull apart (Fig. 6, *B*). Long connecting strands persist for some time (Fig. 6, *C*) as in the preceding species. When these strands are severed it is a common occurrence to find one or both curved back on itself (Fig. 6, *D*) as if they had been suddenly released from a stretching force. As the daughter macronuclei become reorganized in the daughter ciliates the residual chromatin rounds up and degenerates (Fig. 6, *E*), being absorbed into the cytoplasm of that daughter cell in which it lies.

These phenomena exactly parallel those of *Conchophthirius anodontæ* and *C. curtus*.

So far I have never encountered dividing specimens of *C. magna* lacking the residual chromatin, nor have I ever seen any indications of conjugation, either conjugating pairs or reorganizing individuals. This is evidence, I believe, for my above view, that the lack of the residual chromatin in these forms is in some way connected with the reorganization of the macronuclear chromatin following conjugation, a view more firmly substantiated in the case of *C. mytili* (Kidder, 1933*a*).

A very few abnormal divisions were encountered where the nuclear apparatus lagged in its division behind the rest of the cell (Fig. 7, *E*). In these cases the spindles and macronuclei appeared normal, except for their position in relation to the division plane, while cytoplasmic reorganization was far in advance.

Discussion

In reviewing the literature on micronuclear mitosis in ciliates one is at a loss to decide just what type of chromosomal division takes place in the majority of forms. It would seem that either transverse or longitudinal occurs even in closely related species. Of course if one is to accept the contention of Calkins (1930*a*) that within the protozoan nucleus there is only one type of gene per chromosome, then this situation would lose its significance. As Calkins points out, it would then be immaterial whether the chromosomes divided longitudinally or transversely.

To review briefly a few of the cases on record one must note that Stevens (1903) says that the four vegetative chromosomes in the micronucleus of *Boveria subcylindrica* divide transversely. This statement is repeated in a later paper (Stevens, 1910). Her figures indicate a pinching in two in the central portion of each long chromosome.

Calkins (1919) says that the vegetative chromosomes of *Uroleptus mobilis* line up with their long axes parallel to the long axis of the spindle. His figures clearly show this condition. But as to the actual division he says "Whether these rods are divided transversely or longitudinally cannot be determined owing to their minute size and densely packed condition" (p. 306).

In the case of *Uroleptus halseyi* the micronuclei are extremely large and, according to Calkins (1930*a*), the vegetative chromosomes divide in a transverse manner. His figures do not show the actual fission of the chromosomes, however, but merely the metaphase plate and later the two anaphase groups.

In *Conchophthirius mytili* (Kidder, 1933*a*) the sixteen well-formed vegetative chromosomes pull out into dumb-bell-shaped bodies at early anaphase and it certainly appears that division could only have been accomplished in a transverse manner.

Turner (1930) figures the eight vegetative chromosomes of *Euplotes patella* in the early anaphase as dumb-bell-shaped bodies. He says "Whether the chromosomes divide longitudinally or transversely has not been definitely established. Their appearance in the early anaphase would indicate that the latter case were true. As the two halves of a chromosome separate, there seems to be a fine connection between them for a short time which soon breaks. This observation is evidence against, but does not exclude the possibility that the chromosomes split longitudinally. The fact that these chromosomes are never seen in any other position than parallel to the long axis of the spindle indicates that if they do split longitudinally the daughter halves must slip past each other rather than that one or both revolves on the end of the other" (p. 210). This last observation is very interesting in the light of conditions here described for *Conchophthirius anodontæ* where an actual slipping past is demonstrated. Turner's prediction was, no doubt, influenced by his finding of the daughter chromosomes slipping past one another in the third maturation division of *E. patella*.

Manwell (1928) states that the vegetative chromosomes of *Pleurotricha lanceolata* appear to split longitudinally and, in the early anaphase, draw out into V-shaped bodies with connecting bands. His figures indicate only the V-shaped chromosomes in the process of migration. No figure of the actual split is given.

One clear case of longitudinal chromosomal division is given by Chen (1932) in a preliminary report on the mitosis in *Zelleriella.* Here the chromosomes form a rather irregular metaphase plate and plainly divide throughout their length. He states " . . . the longitudinal split of the chromosomes shows clearly and the chromatids or daughter halves of each chromosome can be identified " (p. 270). This species, however, is a member of the Opalinidæ and does not possess the typical dimorphic nuclei of the majority of ciliates and it is, therefore, a comparison of questionable value.

From the above citations it is evident that as far as we know at present the micronuclear chromosomes of ciliates may divide either transversely or longitudinally. In the former method the chromosomes pull into dumb-bell-shaped bodies; in the latter no such dumb-bells are formed.

The chromosomes of *Conchophthirius anodontæ,* and perhaps those of *C. curtus* and *C. magna,* fall into the latter category. The longitudinal split and the slipping past of the daughter halves in the early anaphase have been encountered too frequently in my material to allow for an alternative interpretation. Only future investigations of ciliates possessing large micronuclei with relatively few chromosomes will determine how widespread is this method of chromosomal division.

It is becoming more and more apparent that the macronuclear chromatin is intimately bound up in the reorganization process of the ciliate cell, not only after conjugation and endomixis but during and after binary fission. As regards the extrusion of macronuclear chromatin into the cytoplasm my observations on the three species of *Conchophthirius* described above are parallel to those of Behrend (1916) on *Loxocephalus;* MacLennan and Connell (1931) on *Eupoterion pernix* (figured but not described); Kidder (1933*a*) (1933*b*) on *Conchophthirius mytili* and *Ancistruma isseli;* and recently Haas (1933) on the divisions within the cyst of *Ichthyophthirius multifiliis.* Rossolimo and Jakimowitsch (1929) describe the casting out of granular masses from the seven macronuclei of *Conchophthirius steenstrupii* during binary fission. It seems probable that these masses represent extrusion chromatin although the authors place a rather different interpretation upon them. Their conclusions are based solely upon the staining reactions of these masses after the use of iron hæmatoxylin.

I am inclined to believe that the extrusion chromatin, regularly given off from the macronuclei of the above forms, must represent waste substances of prolonged cell metabolism. Calkins (1930*b*) called the regular extrusion of chromatin from the macronuclei of *Uroleptus halseyi* prior to fission a " purification process." So regular and widespread a

phenomenon certainly cannot be without meaning, and it seems possible that the reorganization process that takes place in the macronuclei of the hypotrichous ciliates is merely a different method of accomplishing the same end—the elimination of worn-out substances.

Summary

1. The nuclear phenomena incident to fission are described for three ciliate commensals of fresh water mussels, *Conchophthirius anodontæ*, *C. curtus*, and *C. magna*.

2. The micronuclear chromatin of *C. anodontæ* forms a granular spireme in the prophase. From this spireme form twelve distinct rod-like chromosomes. On the metaphase plate each chromosome splits longitudinally. In the early anaphase the daughter halves slip past one another and form two groups of twelve chromosomes each. The late anaphase and the telophase are quite regular, the compact daughter micronuclei forming from the twelve daughter chromosomes.

3. The macronucleus of *C. anodontæ* undergoes fission, throwing out a deeply staining ball of chromatin near the division plane. This residual chromatin disintegrates and is absorbed into the cytoplasm.

4. *C. curtus* and *C. magna* parallel *C. anodontæ* in all nuclear activity during fission. The micronuclei are, however, too small to permit the observation of minute details.

5. Several cases of abnormal divisions are reported, occurring in all three species. In these cases it would appear that the synchronization of cytoplasmic and nuclear activity had, in some manner, become disorganized.

6. A short review of the literature dealing with ciliate fission, particularly that concerning micronuclear mitosis and the extrusion of macronuclear chromatin, is given.

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ANALYSIS OF THE REGENERATIVE PROCESSES IN NEMERTEANS

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Because of the sharp demarcation of their tissues, the nemerteans are particularly favorable for a study of the cellular changes which occur during the restoration of the new individual from a fragment of the body. In some species, such as *Lineus socialis,* the regenerative potency is so great that almost any small piece of the body, provided it contains a portion of one of the lateral nerve cords, is able to develop into a minute worm of normal proportions. The persistence of this regenerative ability is demonstrated by repeatedly cutting off portions of partially regenerated pieces until extremely minute individuals less than a hundred thousandth the size of the original are finally obtained (Coe, 1929). The cellular activities involved in such regeneration have already been described (Coe, 1934), and we may now inquire as to the organizing agencies which are responsible for them.

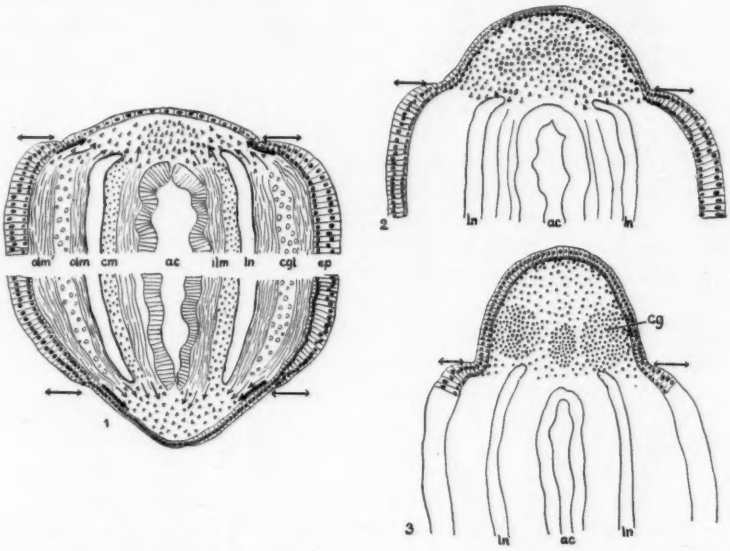
In the regeneration of a fragment of the body of *Lineus socialis* it is evident that the stimulus of the changed internal environment causes a vigorous contraction of the body and a coördinated migration of epidermal and connective tissue cells toward both the cut surfaces. The wounds are thereby closed and healed (Fig. 1).

The cuts also activate the dormant cells which are situated in the parenchyma between the organ systems in all parts of the body. By virtue of their activation these cells assume the properties of regenerative cells. They migrate both anteriorly and posteriorly, finally collecting in large numbers beneath the new epidermis which has already covered the cut ends of the fragment.

Those that migrate posteriorly are soon incorporated into the organ systems of the original body. These organs were injured by the cut and their tissues are in process of restoration, both by the multiplication of the differentiated cells and the incorporation of new ones. The fate of each of the migratory cells is presumably determined by the dif-

ferentiated cells which it reaches. The functional tissues thus organize the new posterior extremity.

Posterior regeneration is therefore closely similar to normal growth, although phagocytosis is more extensive in removing unassimilable elements and more essential in the nutrition of the regenerating tissues than is the case in normal growth.



FIGS. 1–3, *Lineus socialis.*

Fig. 1. Diagram of regenerating fragment of body four days after operation, showing migration of epidermal and regenerative cells toward both cut surfaces as indicated by direction of arrows; *ac*, alimentary canal; *cgl*, cutis glands; *cm*, circular musculature; *ep*, epidermis; *ilm*, *olm*, *olm'*, inner and outer longitudinal musculatures, respectively; *ln*, lateral nerve cord. Double arrows indicate positions of cuts.

Fig. 2. Blastema preceding visible localization of organs; sixth day of regeneration; letters as in Fig. 1.

Fig. 3. Blastema showing localization of prospective cerebral ganglia (*cg*) and (in median line) proboscis and sheath; eighth day of regeneration; letters as in Fig. 1.

The regenerative cells which migrate anteriorly, on the contrary, form a true blastema consisting of an apparently undifferentiated mass of mesenchyme cells (Fig. 2). These cells form the basis of an essentially new individual. The blastema resembles the early embryo in that it seems to be a self-determining system, the new organs being

differentiated without regard to the origin of their cells among the parent tissues. It is at present impossible to state which, if any, of the constituent cells of the blastema are derived from the new epidermis which

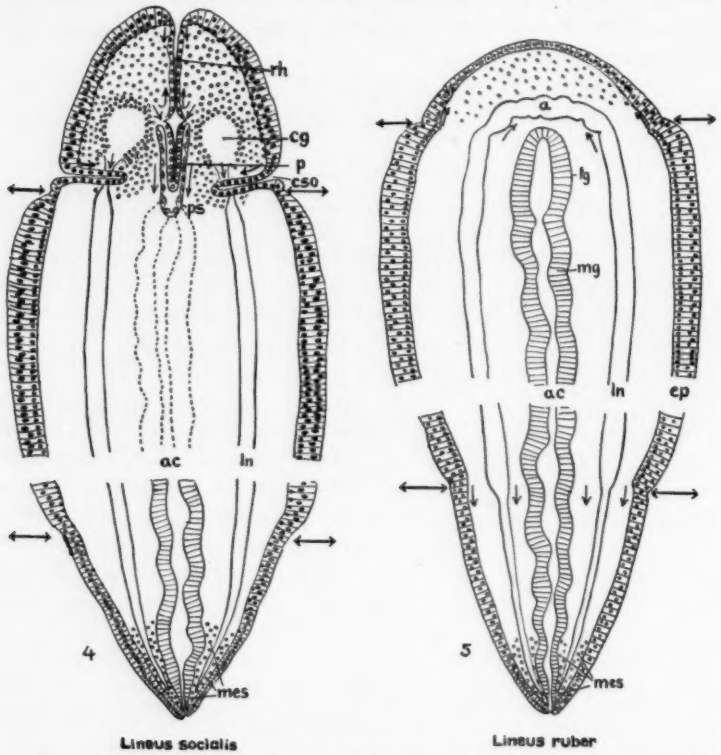


FIG. 4. *Lineus socialis.* Blastema with differentiated primordia of cerebral ganglia (*cg*) with new lateral nerves growing posteriorly to join nerve cords of original fragment; *cso,* invagination of new epidermis to form cerebral sense organs; *rh,* rhynchodeum; *p,* new proboscis growing posteriorly in the new sheath (*ps*). The posterior end shows the elongation of the original organ systems, with migration and incorporation of mesenchyme cells (*mes*). Fifteenth day of regeneration.

FIG. 5. *Lineus ruber.* Portion of body 30 days after operation, showing posterior regeneration only; wound healed at anterior end and nerve cords united, but no blastema is formed; letters as in Fig. 1; double arrows indicate positions of cuts.

covers the mesenchyme. All of the cells appear to be multipotent and capable of differentiation into any of future organs.

Localizations soon become manifest in the blastema and the regen-

erative cells congregate in three principal areas (Fig. 3). Two of these are situated laterally and represent the primordia of the cerebral ganglia, while the median group furnishes the cells for the new proboscis and proboscis sheath.

The cells which are later differentiated into the ganglia are so closely associated with the basal cells of the new epidermis along the lateral margins of the blastema that it seems quite possible that some of them may be of epidermal origin. If so, they may be predetermined or partially determined in the direction of an epidermal derivative such as the nervous system.

Soon after their differentiation the primordia appear to act as induction centers for the other cephalic organs. From the base of the proboscis primordium a group of cells becomes differentiated into a slender tube of epithelium which represents the future rhynchodeum. Simultaneously a group of epidermal cells at the anterior margin of the blastema forms a slender invagination which joins the rhynchodeum. The proboscis is thus brought into communication with the exterior (Fig. 4).

The cerebral ganglia are likewise associated with, and presumably induce, a pair of epidermal invaginations on the lateral margins of the blastema. These represent the future canals of the cerebral sense organs (Fig. 4). The new mouth is formed by another epidermal invagination; this is evidently induced by the anterior end of the new foregut.

As soon as all the organs of the new head have been restored, that is, when the regenerated part has become individualized, it reorganizes all the tissues of the original fragment into units of smaller size. Nutrition by phagocytosis leads to compensatory growth of the new part, the size which is reached before external food is taken being obviously dependent upon the dimensions of the original fragment.

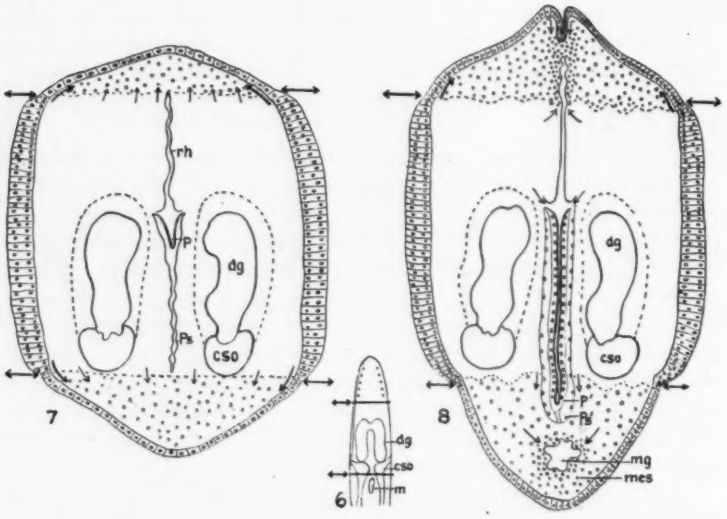
To accomplish this reorganization, the new organs, which have been differentiated in the blastema quite independently of the organs in the original fragment, grow posteriorly and make connections with such of the original organs as are represented in the fragment. The minute new nerve cords join the much larger cords of the fragment, the new mouth joins the old alimentary canal and the new blood vessels unite with the old. Phagocytic activities remove such parts of the old organs as cannot be incorporated into the new and transfer the available materials as nutrition for the new tissues. An essentially new individual of harmonious proportions results.

Since the original proboscis is not represented in the fragment the new proboscis, with its sheath, grows posteriorly into the parenchyma

which fills the space formerly occupied by the old proboscis and sheath or in a corresponding position if the fragment was taken posterior to the end of the sheath. During this elongation the walls of the proboscis become differentiated into epithelial, muscular and connective tissue layers, as previously described (Coe, 1934). Nerves grow into the organ from the new brain.

Regeneration of a New Body from a Portion of the Head

The conditions are somewhat different when a new body is restored from the head alone or even from a portion of the head, as shown in Figs. 6 to 11. If two transverse cuts be made so as to remove the anterior portion of the head as well as the entire body posterior to the



Figs. 6–8. *Lineus socialis.* Early stages in regeneration of new body from portion of head, cut as indicated by double arrows in Fig. 6; *cso,* cerebral sense organ; *dg,* dorsal ganglion; *m,* mouth; *mes,* mesenchyme; *mg,* primordium of midgut; *P,* proboscis; *Ps,* proboscis sheath; *rh,* rhynchodeum; arrows show direction of migrating cells.

cerebral sense organs (Fig. 6), the tissues to be replaced anteriorly will be organized by those remaining. If all trace of the digestive system, including the buccal epithelium, has been removed from the posterior end of the head, an entirely new alimentary canal must be replaced. This is accomplished by mesenchyme cells which migrate posteriorly from the remaining cephalic tissues (Figs. 7, 8). These mesenchyme

cells are preceded by wandering phagocytes which congregate at the site of the future midgut to form an irregular mass of cells with more or less tissue fluid between them. The mesenchyme cells then arrange themselves in a single layer around this fluid area to form the primordium of the midgut (Fig. 9). With further additions and by cell division the mesenchyme cells become differentiated into columnar epithelium characteristic of the digestive system (Coe, 1934).

A median outgrowth of the epithelium at the anterior end of the midgut forms the primordium of the foregut. This is followed by an invagination of the new epithelium on the ventral side of the body to form the new mouth (Fig. 10), exactly as in the case of the regenerating body fragments previously described. The foregut, of mesenchymal origin, then forms a communication with the buccal cavity, of epidermal origin, and the constituent cells are mingled.

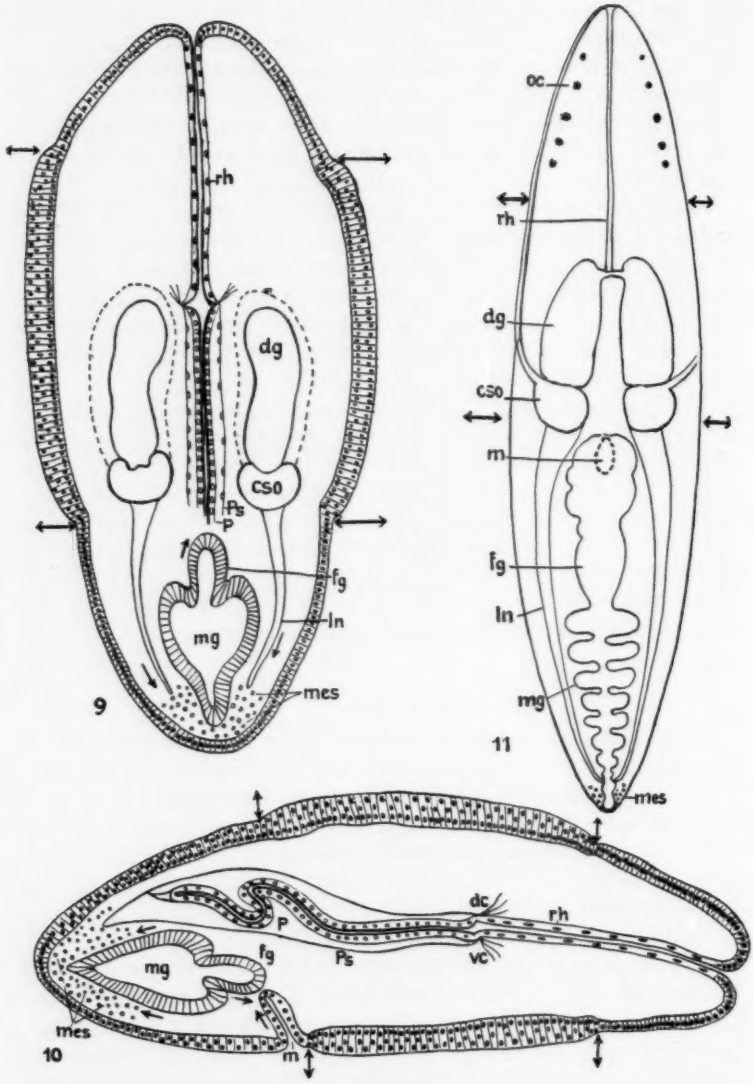
These processes may be interpreted as implying that the epidermal invagination is induced by some stimulus originating in the newly formed foregut, since the epidermal cells appear to be activated only after the foregut has reached a definite stage of differentiation. The posterior end of the midgut is capable of indefinite extension, both by cell proliferation and by the incorporation of mesenchymal cells which are always present at the posterior end of the elongating body (Figs. 9–11).

Reorganization, or regulation, of the old cephalic tissues by phagocytosis supplies sufficient material for the minute new individual which results (Fig. 11).

Regeneration in Other Species

In the genus *Lineus* are three groups of species which differ widely in their capacities for restoring new heads on fragments of the body (Coe, 1929, 1930, 1932, 1934). In one group, including *L. socialis*, discussed on the preceding pages, the ability to restore a new head from a body fragment extends the entire length of the body; in the second group, including *L. pictifrons*, this capacity extends only through the anterior half of the foregut region, while in the third group, of which *L. ruber* ("broad form," Nusbaum and Oxner) is an example, only that part containing the anterior ends of the nerve cords restores a missing head. If we inquire wherein these groups of species differ the answer may be found either in differences of distribution of the regenerative cells or in the agencies which may control their activities.

All three groups show similar capacities for wound healing and for posterior regeneration, and the parenchyma in all these species contains cells of similar appearance. In the first group, however, the cells which migrate anteriorly are immediately organized into the regenerative cells of the blastema and hence of an essentially new organism.



FIGS. 9–11. Later stages in regeneration of new body from portion of head (continuation of Figs. 6–8); *fg*, foregut; *ln*, lateral nerve cord; *dc*, *vc*, dorsal and ventral brain commissures; *oc*, ocelli; other letters as in Figs. 6–8; double arrows indicate positions of original cuts. Compare relative volumes of old and new tissues.

In the third group, on the other hand, such cells as migrate anteriorly retain the characteristics of parenchyma cells and as such supply merely the connective tissues and phagocytic cells for the completion of wound healing but without restoring the missing parts. The growth of these connective tissues may be very extensive and the headless fragment may live for several months. During this period the organ systems may be considerably altered anteriorly, often leading to the fusion of the cut ends of the nerve cords in the median line (Fig. 5). But the old organism remains incomplete and eventually dies, presumably because it lacks either the cells necessary for the restoration of a new head or the suitable stimulus for their activation, or both. The possibility of the presence of an inhibiting influence must also be kept in mind. Since the head alone may restore a new body, while the body itself cannot replace the missing head, and since regeneration does not occur unless portions of the nerve cords are present, we may conclude that the organizing, or morphogenetic, agent is in these species limited to the head and more particularly to the anterior ends of the nerve cords.

The region of complete regeneration in any species would thus be coextensive with bipolarity in the movements of the regenerative cells, extending the entire length of the body in individuals of the first group but only to the posterior ends of the cerebral sense organs in the third. An intermediate condition exists in the second group, where this organizing potency extends from the brain to the middle of the foregut region.

In the first group all parts of the body which contain any portion of the nerve cords are equally capable of regeneration and a hundred simultaneously regenerating fragments may be obtained from a single individual. In the second group only two new individuals have been obtained from one, while in the third group no increase in number has been found possible since only that fragment survives which contains the anterior ends of the nerve cords.

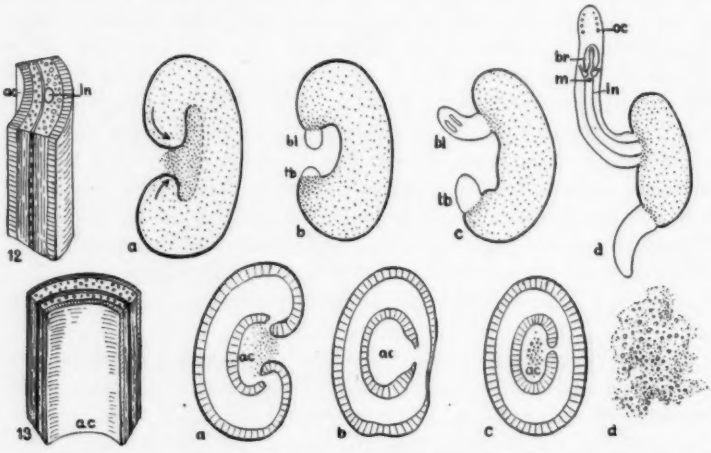
Corresponding differences in the regenerative capacities of different parts of the body in oligochætes are attributed by Stone (1932, 1933) to a restricted distribution of the regenerative cells, while Curtis and Schulze (1934) find that the regenerative ability in species of planarians is correlated with the relative number of regenerative cells preformed in the parenchyma.

Influence of the Nervous System

It must be kept in mind that the processes described appear to be in some way dependent upon the presence of some part of the nervous system. Repeated observation of body fragments of *Lineus socialis*

indicates that at least a small portion of one of the lateral nerve cords must be present in order that complete regeneration and regulation may take place. Wound healing occurs normally in a fragment lacking any portion of the nerve cords and the remaining tissues may be reorganized extensively, but an anterior blastema is not formed. Such reorganized pieces may remain alive for several weeks but individualization is not accomplished and disintegration has followed in every case observed (Fig. 13).

Whether this lack of regenerative capacity is due to the removal of essential regenerative cells or whether to the absence of some stimulus required by the cells remaining is at present uncertain, but from a con-



FIGS. 12, 13. *Lineus socialis.* Fig. 12, regeneration of sector of fragment containing portion of nerve cord (*ln*); *bl*, blastema at anterior end of nerve cord; *tb*, tail bud; *oc*, ocelli. Fig. 13, reorganization, without regeneration, of sector of fragment lacking any portion of nerve cord; disintegration after a survival of thirty days; *ac*, portion of alimentary canal.

sideration of all aspects of the regenerative processes in this and other species the evidence seems to favor the latter alternative.

The extent to which this stimulating agent is present along the length of the nerve cords in various species may, conceivably, be responsible for the observed differences in their regenerative capacities. For we must recall that in other, apparently closely related, species a new head is not formed even when the entire nervous system posterior to the cerebral sense organs is intact. Such differences in the regenerative capacities of morphologically similar species throws additional light on this problem of determination.

In fragments capable of regeneration, the blastema invariably makes its appearance at the anterior cut ends of the nerve cords. In fragments consisting of an entire transverse section of the body, the ends of the two nerve cords are brought closer together by the contractions of the musculatures near the cut surface, while in sectors of the body the single nerve cord ends in the recurved anterior end of the fragment (Fig. 12). In both cases the blastema arises in the new plane of symmetry (Figs. 1–3; 12) at the cut ends of the nerve cords because the regenerative cells have migrated to these positions.

In the planarians likewise the blastema usually arises near the anterior cut ends of the nerve cords, although in certain species even fragments cut lateral to the nerve cord show complete regeneration. In such cases, as Beyer and Child (1930) have shown, the head bud usually forms along the median border of the fragment adjacent to one of the lateral branches of the nerve cord or a group of such branches. They regard the central nervous system as an activating factor concerned in localization, and not as a determining factor nor as necessarily essential for the localization of anterior ends in the planarians studied.

There is a similar close connection between the cut ends of the nerve cords and the position of the regenerative buds in both polychæte and oligochæte annelids. As found by Goldfarb many years ago, removing the nerve cord from near the cut surface causes a considerable delay in the regeneration of earthworms (Siegmund, 1928; Kropp, 1933). If the ends of the cords are turned back and held in this position, normal regeneration does not take place (Bailey, 1930; Holmes, 1931). Evidently the migration of the regenerative cells to the cut surface is thereby prevented, although wound healing occurs as usual.

The inhibiting effects of X-rays on regenerative ability in various groups of animals appear to be due to the destruction of the regenerative cells or to changes which render them incapable of activation. Proliferation of the specialized tissues may also be checked, but wound healing is not prevented unless the dosage is very strong (Zhinkin, 1932; Stone, 1932, 1933).

The bilateral symmetry of the blastema in both planarians and nemerteans is not strictly dependent upon the symmetry of the original fragment, for a fragment taken from either the left or right side of the body produces a blastema of normal symmetry (Fig. 12). The migrating regenerative cells are evidently disposed with reference to the new plane of symmetry imposed by the group as a whole. An asymmetrical blastema has been found only when the original blastema has been split lengthwise. If the parts are then kept separated or one of them removed the original symmetry is slowly restored. Splitting the anterior

end of the fragment before regeneration has made much progress causes the formation of a normal blastema on each part (Coe, 1930).

In the case of wound healing as well as in posterior regeneration the making of the cut causes a disturbance of the normal interrelations of the remaining cells, together with changes in their internal environment. The cells are thereby activated and respond adaptively to the new conditions, finally becoming organized into the functional tissues. The situation at the anterior cut surface is at first similar but complications soon arise because the fragment is without individualization. In addition to the necessity of repairing the organ systems represented in the fragment, a head with entirely new organs must be provided. Head formation resembles embryonic development in that the new organs become differentiated from groups of multipotent cells. In regeneration these cells are derived from dormant parenchyma cells which migrate forward to form the blastema as previously described.

In the activation of these cells it may be supposed that the cut nerve cords liberate some influence, not improbably a growth-stimulating substance, which acts specifically upon the dormant cells of the neighboring parenchyma, transforming them into active regenerative cells and directing their movements anteriorly.

Given this initial stimulus the multipotent cells arrange themselves or are arranged into an aggregate endowed with the power of self-determination. The differentiated primordia of the organ systems act as induction centers for the associated parts of the new systems, whereby the descendents of the original regenerative cells encounter one organizing factor after another in the series of regenerative processes discussed in the preceding portion of this paper. Each step in the series induces the one that is to follow as the organization of the essentially new individual proceeds.

Summary

1. An attempt to analyze the organizing potencies in the regeneration of fragments of the body in several species of nemerteans leads to the conclusion that the cut nerve cords liberate an agent which activates the dormant cells of the parenchyma and transforms them into regenerative cells. Bipolar migration of these cells leads to complete regeneration.

2. The different regenerative capacities in closely related species may be dependent upon differences in the extent of distribution either of this activating agent or of the regenerative cells. In one group the entire length of the body is included and all parts are equally capable of regeneration; in another group the regenerative potency reaches only to the middle of the foregut region, while in a third group of species it is

limited to the anterior ends of the nerve cords and head-formation is limited to a single transverse plane.

3. The blastema is considered to be a self-determining system comparable to that of the early embryo. The constituent cells are evidently multipotent and capable of differentiation into any of the new organs. Once activated they and their descendants complete the regenerative processes.

4. The primary organization center is evidently associated with that part of the nerve cords which is capable of activating the regenerative cells and of controlling their bipolar migration. Secondary organization centers result as soon as the primordia of the organ systems have become differentiated in the self-determining blastema.

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(A more complete list of papers dealing with regeneration in nemerteans may be found in Coe, 1929, 1930, 1932, 1934.)

REGENERATION OF THE TAIL-FINS OF FUNDULUS EMBRYOS[1]

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That the fins of fishes possess the power of regeneration was first demonstrated by Broussonet (1786). From his experiments two observations are worthy of note in connection with this study. First he records that regeneration takes place more rapidly in young fish than in old and that the rate of regeneration may differ from one species to another. Secondly he states that the presence of the stumps of the fin-rays is necessary for regeneration and that in their absence regeneration will not take place.

Fraisse (1885) and Weismann (1892) state that there is very little power of regeneration in the fins of fishes. It is now a well-established fact, as pointed out by Morgan (1902), that many kinds of fish belonging to widely different families will regenerate their tail-fins.

The earliest recorded experiments on the tail-fins of fishes from the standpoint of morphogenesis were performed by Morgan (1900, 1902, and 1906). Later Morrill (1906), Scott (1907 and 1909), Beigel (1912), and Nabrit (1929 and 1931) performed experiments along this same line.

Broussonet (1786), Morgan (1906), and Morrill (1906) agreed that for regeneration to take place the ray stumps must be left. Morrill went so far as to suggest that regeneration does not take place even when the ray stumps are too small; on the other hand, Nabrit (1931) found that the ray stumps are not necessary for regeneration and that if the rays are entirely picked out new rays will appear under the influence of the articulating portion of the basal plate.

After comparing the findings of Harrison (1918) and Detwiler (1918) in *Amblystoma* with the results of his experiments on fish, Nabrit (1929) suggested that a possible similarity existed between the production of limbs in *Amblystoma* and of tail-fins in fishes. Both cases seem to be independently differentiating mesenchymal systems.

[1] I am particularly indebted to Professor S. M. Nabrit of Atlanta University for suggesting this problem and to Professor J. W. Wilson of Brown University for his valuable assistance in the preparation of this paper.

The experiments which form the basis of this paper were planned for three purposes:

1. To examine further the relationship of the ray stumps to regeneration of the fin-rays.

2. To obtain, if possible, new data on the rôle of the basal plate in regeneration of the fin-rays.

3. To compare the regeneration process in the embryo with facts already recorded for the adults.

These experiments were started at the Biological Laboratory at Cold Spring Harbor in the summer of 1931 and were completed there in the summer of 1933. Fixed preparations of the material were studied at Arnold Biological Laboratory, Brown University.

Material and Methods

The material chosen for these experiments was embryos of *Fundulus heteroclitus*. These embryos were obtained from the eggs before the time of hatching by the method devised by Nicholas (1927). The eggs were permitted to develop until the desired stage was reached and were then removed from the chorion. Great care was taken in this removal because the slightest pressure exerted on the eggs will cause injury to the embryo or yolk sac which will render the animal unfit for experimental purposes.

Embryos which are injured show the effect very soon after their removal from the chorion. After a few hours to see if the embryos had been injured in their removal they were ready for operation. Because of their small size the embryos must be operated on under the microscope and therefore it was necessary to employ a method whereby small bits of tissue might be removed. For this purpose Nicholas' (1927) modification of Spemann's technique was used. The cut was made by drawing the tissue to be removed into the lumen of a very fine pipette and carefully cutting the tissue against the tip of the pipette with a spear-point blade. In some cases, especially in embryos just about to hatch, it was found best to remove the parts by supporting the embryo with an ordinary dissecting needle and making the cut with a spear-point blade. In either case great care must be taken not to press the embryo against the bottom of the dish as this invariably results in death. The embryos lack movement and therefore anesthesia is unnecessary. In order to stop the bleeding which occurs when the cuts were made well up into the body, it was found best to transfer the operated embryos immediately to cold sea water or to a cold sodium chloride solution that was isotonic with sea water. The latter was found to be more satisfactory.

Throughout the course of the experiment the embryos were kept in small dishes and the water changed twice daily. No attempt was made to control the temperature as during the summer it was fairly constant in the laboratory, varying only from 18° to 21° C.

Observations were made mainly on living specimens which were placed in a small drop of water on depression slides and examined under a microscope with a 32 mm. objective. A few individuals were fixed in Bouin's fluid and preserved in alcohol for microscopic examination. These were stained in toto with alizarin and cleared in oil of wintergreen. Camera lucida was employed for recording the regeneration process.

Experimental

Preparatory to experimentation a study was made of the normal process of formation of the tail-fins and of the articulating plate. Nabrit (1929) describes the development of the tail-fins as follows: (1) there is a primitive natatory fold; (2) the mesenchymal mass from which the fin-rays and articulating plate develop forms between the end of the notochord and the extremity of the fold; (3) from this mesenchymal mass central rays are the first to differentiate; (4) at the time of differentiation of the first rays the natatory fold is further from the base of the notochord in the central region than in the dorsal and ventral regions; (5) in individuals that hatched in sixteen days the streaking of the rays begins on the seventh day; (6) the additional rays are added dorsally and ventrally but the blood vessels that pass between the rays may be seen to loop in the paths of the rays before the rays are vitally stainable with Nile blue sulphate or with alizarin after fixation. That the rays are present before they can be seen in stained preparations may be demonstrated by crushing the tail under a coverslip and observing the process of fragmentation. By the time the anlage of the first rays to develop are stainable, they are no longer connected with the mass which develops into the basal plate but form an articulation with it (Fig. 1). While my observations confirm Nabrit's in general, there is some doubt as to whether or not the rays and articulating plate develop from the same mesenchymal mass. Soon after hatching the most caudal of the vertebral spines enlarges and becomes incorporated in the plate (Fig. 2). Later the next spine anteriorly does the same and this process is continued until the adult pattern of the tail is formed (Fig. 3).

The experimental animals are divided into three groups dependent upon the mode of operation employed: (1) splitting of the tail-bud; (2) removal of the entire tail; (3) removal of part of the tail.

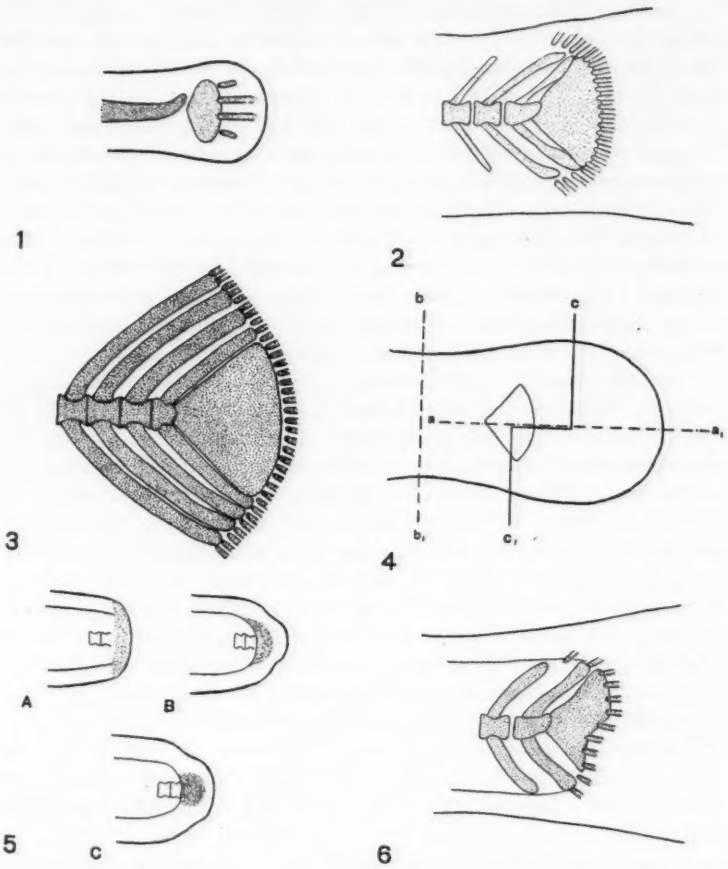

FIG. 1. Diagrammatic sketch of the tail-fin anlage just prior to the time it becomes stainable. The largest rays are the first to separate from the mass and articulate with the plate.

FIG. 2. A camera lucida drawing of the tail at the stage of development when the most posterior of the vertebral spines become incorporated in the plate. (Four days after hatching.)

FIG. 3. A camera lucida drawing of the basal plate of an adult showing the relationship of the vertebral spines to the articulating border of the plate.

FIG. 4. Diagrammatic sketch showing the relationship between the basal plate and the type of cuts made in the various experiments.

FIG. 5. Camera lucida drawings made at various stages after removal of the entire tail-fin anlage. (*A*) twenty-four hours after operation; (*B*) six days; (*C*) twenty-five days. See text for explanation.

FIG. 6. A camera lucida drawing showing the articulation of the regenerated rays with the exposed edge of the basal plate in the ventral region of the tail.

1. The tail-fins of embryos from seven to fifteen days old were deeply incised as nearly as possible through the middle of the fins and basal plate in a posterio-anterior direction, the cut being made along the line a–a_1 of Fig. 4. Such an incision divided the fin, the plate, and a portion of the posterior part of the body into dorsal and ventral parts. The operation is of such a drastic nature, so long an incision being made, that many of the animals operated upon in this manner died soon after the cut was made. The survivors were kept from ten to twenty days and at the end of this time all of the cut plates appeared to have fused together, though in some cases development of the tail was somewhat retarded. The wound usually left a distinct scar or groove running across the tail-bud, which, however, was obliterated after several days. In no case did the operation result in reduplication.

2. Tails were removed from embryos in all stages of development from the fifth day, at which time there is no visible differentiation of the fins or plate, until the time of hatching. The cut was made just anterior to the region of the basal plate and never as far anterior as the position of the dorsal and ventral fins, the cut being made along the line b–b_1 of Fig. 4. In about twenty-four hours after operation the wound heals and by this time there appeared some darkly staining tissue at the level of the cut (Fig. 5, A). By the sixth day this darkly staining tissue had rounded into a small nodule and had taken a place at the end of the spinal column (Fig. 5, B) where it remained as long as the fish was allowed to live without showing any tendency to undergo further differentiation. The mass at the end of twenty-five days is shown (Fig. 5, C) and is the same in appearance as on the sixth day.

In none of the embryos operated on in the above manner did any signs of regeneration of the plate or fins appear up to and including the sixty-fifth day after operation. The stage of development at which the operation is performed seems to have no bearing on the results, in all cases the process was apparently the same. It is doubtful whether regeneration of the plate or fins would ever occur after this type of operation but at present it can only be stated that regeneration fails to occur in the allotted time. Some experiments were carried out on young hatched *Fundulus* of unknown age (measuring from one to two centimeters in length) and the same results were obtained as above with the exception that no nodule was formed at the end of the spinal column. These older animals did not survive as well as the embryos, for death usually ended the experiment between the twentieth and thirtieth day after operation. The cause of death of these fish was apparently a sloughing-off of the tissue starting at the level of the cut, this sloughing process proceeding anteriorly until death took place.

3. Embryos twelve to fifteen days old, at which time the rays and plate are well differentiated, were operated upon so as to remove the ventral half of the plate and all of the rays distal to it, while in the dorsal half of the tail only the distal half of the rays were removed, the cut being made along the line c–c_1 of Fig. 4. At this time there were from six to ten rays articulating with the basal plate. The animals were allowed to regenerate and at the end of from fifteen to seventeen days some were fixed and stained with alizarin. In the ventral half of the tail a portion of the basal plate had been removed prior to differentiation of some of the rays and there was also removed a part of the basal plate with which some rays were articulating. Under the influence of the exposed edge of the basal plate, rays differentiated in the mass of regenerating tissue. These rays were similar in every respect to the other rays except for their more anterior articulation. These new rays articulate with the exposed edge of the basal plate (Fig. 6). This is true regardless of whether the part of the plate they articulate with is derived from spines or from the original central plate. In the latter case rays come out from the border of the plate even though it is not at the level of the unoperated portion of the plate. The shape of the plate may in this way influence the shape of the tail because, after reaching a definite size, the rays cease to grow in length and the tail remains somewhat shorter on the operated side. Regeneration of the plate is a relatively slow process as compared with the rays and in the length of time that the majority of the animals were under observation, usually seventeen days, the plate had not returned to its original size or shape (Fig. 7). In a much longer period, namely fifty days, it was found that the plate had almost completely replaced itself and at this time the tail ceased to be asymmetrical. So far as general observation reveals, the developmental processes of all other parts of the fish are normal.

Discussion

Concerning the extent of regeneration that will take place in the tails of fishes there seems to be, according to the literature, quite a diversity of opinions. All of the papers concerning this phase of the problem cannot be discussed but only the more outstanding ones will be mentioned.

Nussbaum and Sidoriak (1900) have demonstrated that *Salmo fario* operated on the day after hatching would regenerate the posterior part of the body in about ten weeks. If the cuts were made anterior to the anus a new posterior opening is established as well as a new opening for the urethra. After ten weeks fungus usually caused death of the culture.

Duncker (1905), using *Syngnathus*, demonstrated that when the entire tail is removed a perfectly normal one is regenerated. Also, if part of the vertebral column is cut out, accessory tails are produced at the point of injury. However, he was unable to obtain the same results on any other forms used.

The difference between the results obtained from the experiments recorded in this paper for *Fundulus* embryos and those recorded by the above investigators for *Salmo fario* and *Syngnathus* is not apparent at the present time but suggests an explanation on the basis that different species show quite different powers of regeneration.

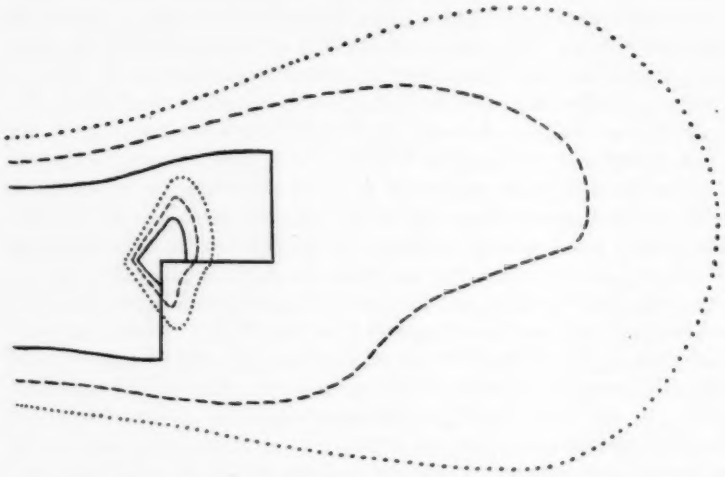

FIG. 7. A composite diagrammatic sketch showing the relative amounts of plate and fin material replaced at different stages after operation. The solid line represents twenty-four hours, the broken line twenty days and the dotted line thirty days after operation.

In a *Fundulus heteroclitus* embryo which was cauterized just posterior to the body cavity prior to tail-fin differentiation, Nabrit (unpublished results) has obtained an abnormal tail with four rays. The fish died soon after hatching. It was not possible to determine the exact origin of these rays. They appeared, however, in the center of the posterior surface. The fish did not regenerate the lost part of its body. There is no definite indication that regeneration will ever take place after such an operation but rather the undifferentiated mesenchymal bud of the dorsal and ventral fins may differentiate at the end of the animal and simulate a tail.

The observations recorded in this paper on removal of the entire tail-fin anlage indicate that the factors responsible for regeneration do not extend more anterior than the region of the basal plate. The experiments on removal of parts of the plate reveal that the rays can be regenerated even before the plate has completed itself. These results indicate that the regenerative factors, be they nervous system, distance from the base of the tail, axial gradient of metabolism, or other factors, are segregated in the plate-fin region early in the process of differentiation. In fact, this type of segregation apparently takes place before any visible differentiation has taken place. This observation confirms the work of Nicholas (1927), who has shown that *Fundulus* embryos undergo early in their development an extremely high degree of differentiation. Such a fact in itself may explain in part the lack of power of regeneration of certain parts. That splitting of the tail does not result in reduplication is a result to be expected considering the extremely high degree of differentiation in these embryos. Such results as reported above are not confined to *Fundulus* embryos as similar results have recently been obtained by using young sunfish and guppies.

It has been demonstrated that if all of the ray material and part of the basal plate is removed, the basal plate will replace itself and that rays will be differentiated in the regenerating tissue under the influence of the exposed edge of the basal plate. This fact indicates conclusively that the presence of ray stumps is not necessary for regeneration of the rays to take place.

Harrison (1918) and Detwiler (1918) have shown that in *Amblystoma* the fore-limb develops from a self-differentiating and equipotential system. The limb anlage therefore is an entity, which, except for its dependence for nourishment, is independent of its surroundings in the attainment of specific form. Detwiler showed that it was possible to initiate the development of a limb in almost complete absence of the shoulder girdle. They suggest that the limb anlage may be regarded not as a definitely circumscribed area, like a stone in a mosaic, but as a center of differentiation in which the intensity of the process diminishes as the distance from the center increases until it passes away into indifferent regions. This conception suggests that the growth of the limb may be controlled by the distance of the part from the center of the anlage. If the anlage is extirpated other mesenchyme could, however, in a restricted sense, simulate the extirpated anlage and produce an apparently normal limb. This explanation is based on the conception of the mesenchyme as a formative factor in growth and, according to Nabrit (1929), the same explanation may be offered for the type of regeneration obtained in the tails of fishes.

It is concluded, therefore, that regeneration of the tail-fins of fishes and the same process in the limbs of *Amblystoma* are similar. The basal plate is apparently like the shoulder girdle in regenerative capacity, since it may replace itself and give rise to or induce the development of rays. Hence the rays, like the limbs of *Amblystoma,* and the basal plate, like the girdle, belong to a self-differentiating mesenchymal system.

Summary

1. A study was made of regeneration of the tail-fins and basal plate of *Fundulus heteroclitus* embryos operated upon prior to the time of hatching.

2. Splitting of the tail-fin anlage does not result in reduplication of the tails.

3. Removal of the entire tail-fin anlage results in no regeneration up to and including the sixty-fifth day after operation.

4. If part of the basal plate is removed with the rays distal to it new rays will appear in seventeen days and the plate will regenerate in fifty days.

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PRESENT STATUS OF LONGITUDINAL DIVISION IN HYDRA

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Longitudinal division in *Hydra* has long been considered a method of reproduction, but it was admitted that it was rare in occurrence. The author wishes to point out works which show that the process is merely one of regulation and not of reproduction, and to substantiate this view with some of his own experience.

Several authors have described longitudinal division, Trembley (1744) probably being the first; but it should be noted that Baker, working at the same time as the Swiss priest, also noted this phenomenon. Jennings (1883) records a similar process as do also Koelitz (1909) and Ross (1914). These last three authors have merely described the process of division and have not entered greatly into discussion of it. Trembley noted that the division could be instigated by cutting the oral end of the animal.

Marshall (1882) found that if the anterior end of a hydra were partially split in two, each half became a distinct anterior end. The body then began to separate slowly into two parts, the division taking place at the angle between the two oral ends until the two parts became completely separated. King (1901) repeated the experiment in a large number of cases with practically the same result. The work of these men and others shows beyond a doubt that hydra can be stimulated to divide longitudinally.

Morgan, in his memorable book on regeneration, quotes von Kennel as asking the questions, " Can accidental injuries account for the result (viz. for the division in *Lumbriculus,* planarians, and starfish), since how few starfish are there with regenerating arms in comparison with the enormous number of uninjured individuals? Should we not rather look for the external stimuli that have initiated the process of self-division? "

Morgan gives his own opinion on the subject when he says, " Hydra appear rarely, if at all, to divide by a cross-division, and, although one or two cases of longitudinal division have been described, it is not improbable that they have been started by the accidental splitting of the oral end." More recently Hegner (1931) states that the longitudinal

division is the result of the animal's readjustment to release itself from an abnormal condition.

During the writer's recent work on regeneration (Roudabush, 1933), it was his privilege to make a series of observations which will lend some evidence toward the views of von Kennel, Morgan and Hegner. It was noted that during the process of turning hydra inside out, a number of individuals were torn at the anterior end, and since these were not useful for the problem then at hand, they were isolated in other culture dishes and examined at intervals to note just what would be the outcome of such injury. A large number of these injured specimens regenerated into normal individuals while a smaller percentage was found to develop two anterior ends and eventually complete the division. Thus a large number of dividing specimens was seen and all were begun by some injury inflicted on the anterior end. No notes were kept on these discarded animals, so no relationship can be definitely drawn between the number injured and the number showing division.

More recently a good stock of animals was secured for the purpose of obtaining a percentage relationship between those which showed division and those which did not. Both *Pelmatohydra oligactis* (Pallas) and *Hydra vulgaris* Pallas were used in this experiment. The animals were cut, or rather torn, through the mouth to a distance just posterior to the base of the tentacles. The distance was practically the same for every specimen so as to eliminate the possibility that some would have greater stimulus than others to divide. These animals, after having been cut, were placed in small dishes and examined daily until all the apparent injuries had healed. Those with two oral ends and slightly divided bodies were judged to show longitudinal division. These criteria were chosen merely because if such a condition were found in nature the specimen would be promptly judged to be undergoing division. The author realizes that some of these may never have completed the division either because of depression ensuing or because of the absorption of one of the anterior ends, but it still remains true that such specimens if found would have been considered to show division.

Sixteen and nine-tenths per cent of the animals treated as described showed the evidence of division. This percentage, doubtless, would have been much higher had the animals been torn farther down the body. (It should be noted that 11 per cent of the hydras which did not show division had an increase in the number of tentacles; most of them increasing only by one, but several by two.) This percentage may vary with hydras taken from different conditions because their ability to regenerate depends upon their past history. The fact that the percentage is low does not affect its significance.

The literature itself is evidence to the fact that longitudinal division occurs very rarely or at least it is rarely recorded. The facts discussed above show why this should be. If a number of hydras should happen to become injured at their anterior ends, only about 17 per cent of this number would regenerate two oral ends and undergo division. Of this 17 per cent, only a very few would ever fall into the hands of a scientific observer and so the number of records necessarily would be low. These records would also be lowered if the number of injured hydras were by any means kept low.

The foregoing statements are offered as an answer to von Kennel's first question. As to his second, let us consider how hydras could become injured in nature in such a manner as to cause division.

Since it is the mouth which is primarily involved, is it not conceivable that, should the animal attempt to take in pieces of food—worms or crustaceans—which are too large, this act would tear its mouth and thus instigate—at least in some cases—the division?

The author has seen such activity both in aquaria and in animals taken from their native pools. One case is of particular interest since it was watched through nearly the entire process.

While examining newly caught hydras one day, it was noted that one was attempting to swallow a worm which was nearly as large as the hydra itself. The worm, needless to say, was still struggling even though it was half inside the hydra. The worm was pushing against the side of the mouth of the hydra, as if in an effort to pry itself free. Other duties made it necessary to set these animals aside for a short time and when they were again observed the worm had in some manner released itself and in the process had split the hydra through the mouth. As the observation continued, the hydra developed two oral ends and showed typically longitudinal division as described above.

Since this answers von Kennel's question, and shows that the division can be caused by an external stimulus rather than by an internal condition, it is highly probable that it is a process of regeneration or regulation rather than one of reproduction.

Summary

1. It is concluded that longitudinal division in *Hydra* is not a reproductive process but is one of regeneration.

2. Longitudinal division is stimulated by the tearing of the hydra's mouth.

3. About 17 per cent of those animals torn undergo division; the remainder merely heal the cut.

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THE INFLUENCE OF TEMPERATURE UPON THE AMYLASES OF COLD- AND WARM-BLOODED ANIMALS

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The temperature relations of the enzymes of cold-blooded animals are a subject of considerable interest. Apparently these enzymes act most efficaciously at temperatures seldom or never attained by the living animals. It has not been established irrefragably that the enzymes of the poikilotherms are adapted to act at lower temperatures, *i.e.* that the temperature coefficient is so small as to make the enzyme about as effective at low temperatures as at high.

The best way to study the relation between temperature and the activity of an enzyme is to study the temperature coefficients over a wide range, with rigid control of as many factors as possible. This has not been done adequately for representative animals of different vertebrate classes. A great many of the experiments which have been done have been carried on without any regard for pH, ionic concentrations, digestion periods, or enzyme-substrate ratios. As Haldane (1930) remarks of temperature coefficients of enzymes generally, "Many of the data in the literature are clearly unreliable." Kendall and Sherman (1910) showed how little a temperature coefficient may mean if the experimental conditions are not regulated properly; in the absence of electrolytes, they found the optimal temperature for amylase to be 20° C., but with added NaCl, the activity at 40° C. is four times as great as at 20° C.

It is the purpose of the present investigation to compare the temperature relations of amylases of representative cold- and warm-blooded animals. The temperature coefficients for the amylases were determined between 3.5° and 65° C., with rigid control of the factors just mentioned. These data should answer the questions of whether the amylase of the cold-blooded animal is adapted to act at lower temperatures, and if it has a lower optimal temperature than the amylase of the warm-blooded animal.

Incidentally, data will be presented which show that the "optimal

temperature" is influenced by the period of digestion and by the enzyme-substrate ratio.

In Table I is given a summary of some of the literature bearing upon the problem of temperature adaptations of cold-blooded animal enzymes. For the reasons given above, many of the recorded results can not be accepted as valid and consequently many papers are not cited. [The literature up to the beginning of the present century is reviewed by Yung (1899); Sullivan (1907) provides an English equivalent.]

It is of interest to compare the temperature coefficients found for amylases of different origins with those to be reported here. The literature is tabulated in Table II; the coefficients were calculated from the data given, as in most cases the Q_{10} was not given by the authors.

TABLE I

Temperature adaptations reported for digestive enzymes of cold-blooded vertebrates

Investigator	Date	Source and enzyme	Adapted to lower temp.	Lower optimum than mammalian
Riddle	1909	Fish, amphibian and reptilian pepsin	Yes	—
Rakoczy	1913	Pike pepsin	Yes	Yes
Hosaka	1918	Frog amylase and trypsin	Yes	Yes
Müller	1922	Frog pepsin	No	No
Kenyon	1925	Pickerel pepsin	Yes	Yes
		Fish, amphibian and reptilian pepsin	No	No
Oshima and Sasaki	1925	Fish pepsin	Yes	Yes
Oya and Harada	1926	Fish amylase	—	No
Yio	1931	Fish amylase	Yes	—
Pjatnitzkij	1931	Frog pepsin	No	No

MATERIAL AND METHODS

The amylases of human saliva, terrapin pancreas, and fish pancreas were studied, using the copper reduction method described by Mathews (1920) and two iodine methods detailed below. All three methods were used simultaneously. The temperatures at which the experiments were made were: $3.5 \pm 1°$, $15 \pm 1°$, 25°, 35°, 45°, and 63° C.—the last four being constant to 0.05° C. Eight to ten experiments were made with each enzyme.

Preparation of Enzyme Solutions

Human Salivary Amylase.—After washing the mouth several times with water, the flow of saliva was stimulated by chewing on paraffin.

The saliva was collected in a beaker. To facilitate accuracy and rapidity in pipetting, the saliva was diluted with seven volumes of distilled water; it was used at once.

Reptilian Amylase.—The amylases of two reptiles were used: *Terrapene carolina* (Linnæus) and *Chrysemys* sp. The pancreas was removed from the freshly killed animal, rinsed, blotted and ground with glass fragments. The mass was suspended in 50 per cent alcohol for twenty-four hours. The alcohol-tissue ratio was adjusted to give approximately the same amylase concentration as was found in the diluted saliva, *as measured at 35° C.*

TABLE II

The temperature coefficients for amylases, recorded in literature

Source	Q_{10}						Author
	0–10°	10–20°	20–30°	30–40°	40–50°	50–60°	
Beef pancreas	3.3*	2.25	1.6	1.0	0.7	0.17	Roberts (1881)
Malt	2.8	1.9	1.6	1.6	—	—	Müller-Thurgau (1885)
Malt	—	—	1.9	1.6	1.3	0.6	Vernon (1901)
Beef (?) pancreas	—	—	2.0	2.0	—	—	Kendall and Sherman (1910)
Malt	—	—	2.2	1.4	—	—	Van Laer (1912)
Potato	—	1.5	1.05	1.04	0.5	0.6	Doby (1914)
Merck's diastase	No temperature effect between 5° and 45° C. Experiments at 5° intervals.						Koenig (1920)
Malt	—	—	1.96	1.65	1.43	—	Lüers and Wasmund (1921)
Human saliva	2.9*	2.2	1.9	1.6	1.2	—	Ernström (1922)
Beef (?) pancreas	—	—	2.1	1.8	1.4	0.77	Cook (1925)
Malt	—	—	1.8	1.6	1.3	0.76	" "

* Simple extrapolation.

Fish amylase.—The pyloric cæca and interdigitated pancreatic tissue of the menhaden, *Brevoortia tyrannus* (Latrobe), were removed and treated exactly as was the pancreas of the terrapin.

Preparation of the Digest

A 2.5 per cent preparation of soluble starch served as substrate. Phosphate buffers, final concentration 0.05 M, maintained pH 6.9. Ernström (1922) has shown that the factor A, of Arhennius' equation, and therefore the temperature coefficient, is independent of pH in the case of amylase. Hence the results with different amylases are com-

parable even though pH 6.9 may not be optimal for all. Sodium chloride was added to give a final concentration of 0.05 M. The final concentration of ethyl alcohol was about 0.22 M (1 per cent). In the fish and reptilian enzyme experiments, the alcohol served as the menstruum for the enzyme; and for the sake of uniformity of conditions, it was added in the determinations with salivary amylase. Of course there is a difference in that the salivary amylase had not been exposed to alcohol for twenty-four hours.

Fifty-milliliter portions of the soluble starch preparation were put into Ehrlenmeyer flasks and allowed to come to the temperature of the respective baths. At ten-second intervals, one milliliter of enzyme solution was added to each of the series in turn. The flasks were shaken to ensure thorough mixture.

Determination of Activity

The Iodine Methods.—The first iodine method consisted in determining the time required for the digestion of the soluble starch to the point at which it just fails to give the blue color reaction with iodine; the endpoint is that described by Wohlgemuth (1908). At intervals of one minute, five drops were removed from the digest and added to 5 ml. N/8,000 iodine solution. (See Johnson, 1908; Chesley, 1931 and 1934.)

The second iodine method consisted merely in continuing the above procedure to determine the time in which no color reaction occurs with iodine. The methods will be referred to as "no blue" and "achromic" iodine methods.

The Copper Reduction Method.—At the end of thirty minutes, a 5 ml. specimen was removed from each of the digests, described above, and run into an excess of Fehling's solution (25 ml. diluted to 95 ml. with distilled water). The mixture was put into a bath at 112° C. and left for exactly 6 minutes. It was then removed and filtered through a Gooch crucible, using suction. The precipitate was washed with distilled water at 60° C., then dissolved in M/2 ferric ammonium sulphate-sulphuric acid mixture and immediately titrated with N/2 potassium permanganate. The amount of reducing sugars can then be calculated from this titration value, and the enzyme activity thus estimated.

Results and Discussion

The Temperature Relations of the Amylases

When the activities of the enzymes are plotted against temperature, very similar curves are obtained for all three amylases, whatever the

method of determination used. (See Fig. 1. From Table III, it is evident that the copper reduction method gives curves similar to those obtained with the iodine methods.)

From the divergent courses of the curves above 35° C., it may be seen that the optimal temperatures for the same period of time are somewhat lower for the cold-blooded animal enzymes than for human amylase. This is, of course, due to heat destruction of the enzyme.

TABLE III

The temperature coefficients for the amylases of the human being, terrapin and menhaden. At 45° C.—"no blue," 10 minutes; "achromic," 25 minutes, digestion. At all temperatures, copper reduction, 30 minutes.

Range	Human salivary amylase			
	No blue	Achromic	Cu. red.	Mean
°C.				
5–15*	2.4	—	—	—
15–25	1.8	1.9	2.8	2.1
25–35	1.7	1.6	1.5	1.6
35–45	1.7	1.6	1.2	1.5
45–63	1.3	1.0	0.9	1.1
	Terrapin pancreatic amylase			
5–15*	3.3	3.4	3.2	3.3
15–25	2.0	2.0	1.8	1.9
25–35	1.6	1.5	1.2	1.4
35–45	1.6	1.6	1.5	1.5
45–63	0.6	—	0.7	0.6
	Menhaden pancreatic amylase			
5–15*	2.7	2.5	3.3	2.8
15–25	2.2	2.0	1.7	2.0
25–35	1.6	1.5	1.3	1.5
35–45	1.0	0.6	0.9	0.8
45–63	—	—	—	—

* Simple interpolation.

From the relative positions of the curves for the "no blue" and "achromic" methods, it is evident that the order of heat inactivation of the amylases is fish > terrapin > human. However, this difference in the behavior of the crude enzyme preparations does not justify the statement often made that the amylases are different. The heat inactivation of the enzyme is probably due to protein coagulation; the proteins generally of poikilothermal animals have lower coagulation temperatures than those of warm-blooded animals. The enzyme may be dragged

down bodily by concomitant proteins while actually little affected by the heat. Also amyloclastic activity may be due to some specific chemical group prosthetic on, or inherent in, the protein molecule and common to amylases of all origins. Yet the behavior of the enzyme would be conditioned by the behavior of the whole complex.

From the origins of the curves, and from the temperature coefficients between 5° and 15° C., it may be seen that the amylase of the menhaden is better adapted and that of the terrapin less adapted to act at lower temperatures than is that of human saliva. In the matter of temperature adaptation, it would be interesting to study the enzymes of arctic

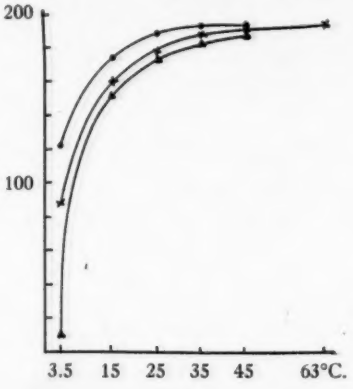

FIG. 1

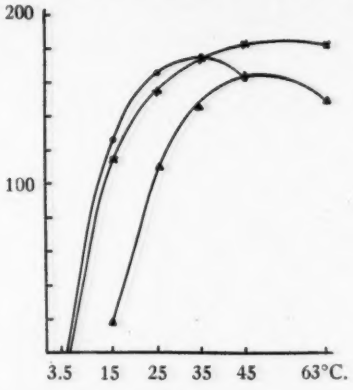

FIG. 2

1. "No blue" method.
2. "Achromic" method. The influence of temperature upon the activities of amylases of cold- and warm-blooded animals. Ordinates: $(200-T)$. T is the time required for the disappearance of the color reaction with iodine. x indicates human salivary amylase; △ terrapin pancreatic amylase; ● menhaden pancreatic amylase.

fishes feeding actively at temperatures near 0° C. Probably the temperature coefficients between 0° and 10° C. are low for such enzymes; this is suggested by the coefficient for menhaden amylase in this range.

The temperature coefficients for the different amylases studied have been calculated and tabulated in Table III. Between 15° and 35°, the coefficients are substantially the same for the three amylases. The variations below and above this range have been discussed above.

The intermediate position of human amylase, in adaptation to low temperature, is of interest in that it does not accord with Riddle's (1909) finding for pepsin. Riddle concluded, from his experiments upon digestion in Mett's tubes introduced into the stomachs, that "higher forms"

progressively lose the power of digestion at lower temperatures. Thus he thought that he had demonstrated an evolutionary series in the development of digestive power.

Factors Influencing the "Optimal Temperature"

A comparison of the curves (Figs. 1 and 2) for any one of the enzymes, obtained by the different methods, shows that the longer the period of digestion the lower the optimal temperature. The "no blue" method covers the least time—ten minutes at 45° C. The optimum, as determined by this method, is about 40° C. for menhaden amylase. The achromic method gives 35° as the optimum for the same enzyme acting in the same digest for an additional fifteen minutes (as determined at 45° C.).

The enzyme-substrate ratio is another factor which will cause the optimal temperature to vary from experiment to experiment unless a constant proportion is set. In the experiments with saliva, the achromic point was reached first in the digest at 63° when the usual concentration of enzyme was used. With half this concentration, the digest at 63° lagged behind those at 45° and 35°. With still less enzyme, the 63° digest never reached the achromic end-point, due to heat inactivation of the enzyme; yet of the series, this was the first to reach the "no blue" end-point. This fact is, of course, a corollary of the fact that the optimal temperature varies with the period of digestion.

It is almost meaningless to speak of the "optimal temperature" of an enzyme without specifying pH, ionic concentrations, period of digestion and enzyme-substrate ratio. This cumbersome changeling will still be without any great significance except to give some idea of the thermostability of the enzyme. While the optimal temperature of an enzyme may be relatively high if the digestion period is short, the optimum will be considerably reduced as the period is prolonged.

Summary and Conclusions

The temperature coefficients for the amylases of human saliva, terrapin and fish pancreas have been determined for the range between 3.5° and 63° C.

Three methods were used simultaneously; a copper reduction method and two iodine methods.

Certain factors were found to influence the temperature effect upon the activities of the amylases. These factors and others were rigidly controlled in the routine experiments.

The conclusions drawn were as follows:

1. *Temperature Relations of the Amylases:*

a. Under comparable conditions, the optimal temperatures of the amylases are, human> terrapin> fish.

b. Fish amylase acts more efficaciously at 3.5° than do terrapin and human amylases. The order is fish> human> terrapin.

2. *Factors Influencing the Optimal Temperature:*

a. The enzyme-substrate ratio has an effect. The larger the proportionate amount of enzyme, the higher the optimal temperature in a given period. This effect depends upon heat inactivation of the enzyme.

b. The longer the period of digestion, the lower is the optimal temperature. This also depends upon the heat inactivation of the enzyme.

I wish to thank the Zoölogy Department of Duke University, and especially Professor F. G. Hall, for the facilities granted me while carrying on this work. A portion of the experiments were done at the United States Bureau of Fisheries Laboratory at Beaufort, N. C. I acknowledge my indebtedness to the Director of the station, Dr. H. F. Prytherch.

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THE STRUCTURE AND REACTIONS OF THE TENTACLES OF TEREBELLA MAGNIFICA W.

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Terebella magnifica Webster is a typical sedentary tube worm found under stones in the shallow water of the Bermuda Islands. Like many related forms it possesses numerous tentacular filaments which function in the building of the tube, obtaining food, respiration, and protection. The remarkable feature of these tentacles is their ability to survive and react after removal from the body of the worm. This automaticity and their length, which often exceeds 50 cm. when extended, provides material for numerous physiological experiments. The present paper deals with certain structural aspects of these tentacular filaments and their reactions to light and electrical stimulation.

Specimens of *Terebella* were obtained in large numbers along the shores of Long Bird Island and were easily kept in shallow dishes in running sea water. When gravel was provided they built new tubes and reacted very much as in normal surroundings, except that when exposed to light the tentacles were always more or less contracted, while in nature worms were occasionally seen with their fully extended tentacles exposed to brilliant illumination. The tentacles, which number from 60 to 100 in a large specimen, may extend to a length several times that of the worm or may be tightly coiled in a mass only a few centimeters in length. Tentacles which had been removed from worms survived for twelve days in bowls of sea water provided the water was changed frequently. New tentacles grew at the rate of 1 to 1.5 cm. per day.

Figure 1 is of a section through the midregion of a tentacle. The greatest diameter is about 1 mm. Along one side extends a deep groove. This is lined with ciliated epithelium and the very active cilia beat constantly toward the base of the tentacle. Microscopic examination of a living tentacle shows a constant stream of small particles consisting of diatoms and other small organisms being swept along this groove toward the basal end. The edges of the groove may be tightly applied one to the other, forming a ciliated tube, or they may be applied by a sucking action to rocks and algæ where the food is swept into the tube.

Covering the exposed surface of the tentacle is an epidermis containing numerous mucous glands (Fig. 3), which are more highly specialized than in the earthworm. Upon stimulation with diluted acetic acid, these glands discharge with considerable force, expelling a stream of mucus which forms a filament several times the length of the cell. The discharged gland resembles very closely a discharged nematocyst.

Longitudinal muscle fibers are most numerous, but in addition there is a complicated system of circular, cross, and oblique fibers. This system of muscles enables the tentacle to execute its many movements.

The central portion of the tentacle is a space, possibly continuous with the cœlomic cavity of the worm, containing a fluid in which amœboid cells are numerous.

The nervous system of the tentacle consists of five longitudinal groups of fibers (Fig. 2) with regularly arranged branches. Two large bundles are located one on each side of the tentacle. A medium-sized bundle is found opposite the ciliated groove and between this and the large lateral groups are two smaller bundles. Fibers enter and leave these bundles at regular intervals by way of circular connections which may be seen on one side of the tentacle in Fig. 2 and in a longitudinal section of a tentacle in Fig. 4. These branches may be traced into the region of the ciliated groove, gradually decreasing in diameter as the distance from the longitudinal bundles increases. Because the only material available for histological study was fixed in Bouin's fluid, it was

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Figures 1–6 are photomicrographs taken at various magnifications.

FIG. 1. Cross-section of a tentacle near midregion.

FIG. 2. Detail of region of tentacle containing the longitudinal bundles of nerve fibers and on one side a circular nerve connective which joins these bundles and extends to the side with ciliated groove.

FIG. 3. The mucous glands of the epidermis.

FIG. 4. A longitudinal section through one of the large lateral nerve bundles showing the segmental arrangement of the circular connectives. (This photograph has been retouched in order to accentuate the outlines of the nerve tracts.)

FIG. 5. Details of the region of the lateral longitudinal nerve fiber tracts showing the characteristic thinning of the epidermis over these bundles.

FIG. 6. Some of the nuclei occasionally seen associated with the nerve fiber bundles. These may be nuclei of sensory cells.

CC = circular connective.
LLN = large longitudinal nerve fiber tract.
MN = median nerve fiber tract.
NU = nuclei of nerve or sensory cells.
SLN = small longitudinal nerve fiber tract.

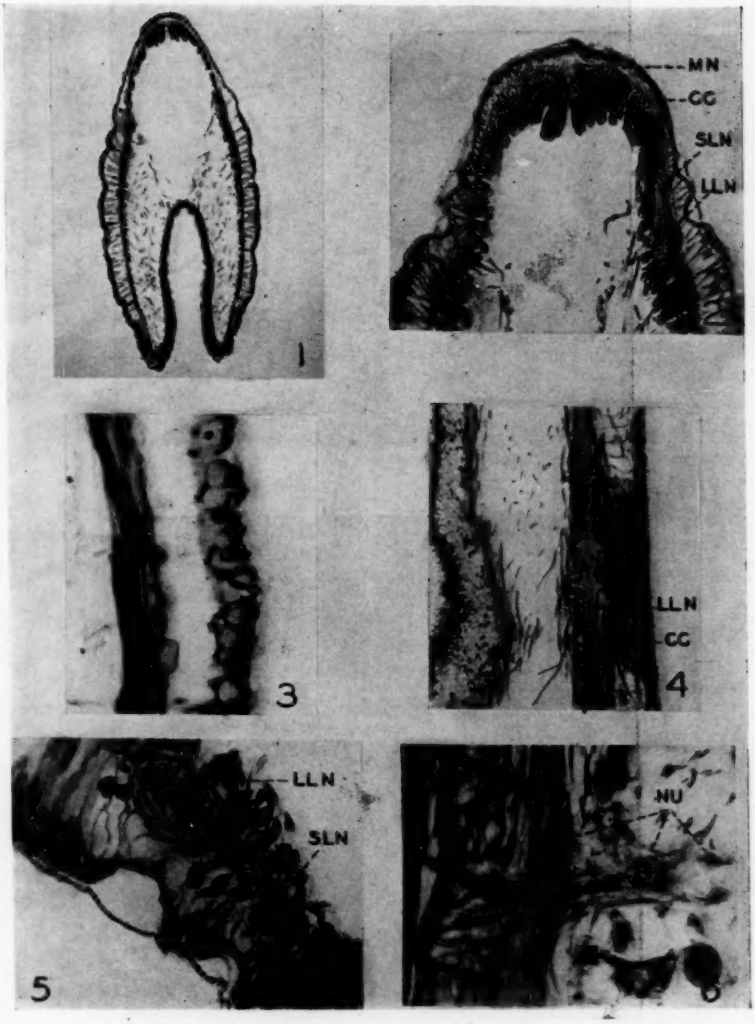
1
MN
CC
SLN
LLN
3
LLN
CC
4
LLN
SLN
5
NU
6

FIG. 7. Photograph of dark-adapted worm taken with a brief flash of light. Note well-extended tentacles.

FIG. 8. Photograph of same worm as seen in Fig. 7 taken few seconds later. Note contracted tentacles.

impossible to trace individual nerve fibers and to locate readily sensory cells. The characteristic thinning of the epidermis over the lateral longitudinal bundles of fibers, seen best in Fig. 5, suggests that the photoreceptors may be located in this region. Nuclei of nerve cells are found in this vicinity and are usually more evident at points where circular connectives join the bundles (Fig. 6). The infrequent occurrence of nuclei within the bundles of nerve fibers leads to the conclusion that few intermediate neurones occur within the tentacle. A study of the responses of isolated and intact tentacles enables one to draw more definite conclusions regarding the functional arrangement of the nervous system of the tentacle.

Reactions of Tentacles to Light

When a specimen of *Terebella* has been in the dark for one-half hour or longer the tentacles are relaxed and extended to full length (Fig. 7). In this condition they are very sensitive to light and illumination with light of a few foot candles in intensity results in a rapid contraction of the tentacles followed by active writhing movements (Fig. 8). The reaction time, even at low intensities of illumination, was found to be too brief to measure accurately with a stop watch but was estimated to average about 0.5 second.

If a tentacle is removed and placed in sea water, in the dark, it behaves as though it were still a part of the animal. It becomes extended and quiescent after a period of dark adaptation and when illuminated reacts in as short a time as does an attached tentacle. The reaction time depends only very slightly on the intensity of illumination and over a range from 5 to 200 foot candles the change could not be measured accurately. It is probable that the use of brief flashes of light as employed by Hecht (1919–1920) in studying the sensitivity of the siphons of *Mya* would have yielded data which might have been studied quantitatively, as the reaction time is doubtless dependent on the length of the exposure period.

When a single attached tentacle is explored with a point source of light, beginning at the base and moving toward the tip, there is seldom a contraction until a portion of the distal third of the tentacle is illuminated. This might be accounted for by assuming a concentration of photoreceptors near the distal end or by assuming a nervous system which conducts primarily toward the base of the tentacle. Further evidence in support of one of these views will appear later.

One other interesting fact regarding the reaction to a point source of light is the independent behavior of single intact tentacles. The illumination of the tip of one tentacle and its subsequent withdrawal is

rarely followed by movement of other tentacles. This indicates little coördination between the many tentacles.

Reaction of Tentacles to Electrical Stimulation

In order to obtain further evidence on conduction within the tentacle, electrical stimulation was used. An isolated tentacle was arranged in such a manner that on either side of the electrodes a portion of the tentacle passed over blocks of paraffin. The remainder of the tentacle was in contact with a glass plate where it was kept moistened with sea water. By means of an induction coil both single shocks and repeated stimuli were applied to a midregion of the tentacle. The lowest stimulus which elicited a response of the proximal half of the tentacle never caused a contraction of the distal half. The distal portion contracted only after repeated stimuli of high intensity.

Discussion

The reactions of isolated tentacles of *Terebella* to light and electrical stimulation indicate that there are probably direct connections between receptors and muscles as demonstrated histologically by Dawson (1920), in the ventral region of the earthworm. The experiments of Moore (1923) and Hess (1925), which confirmed Dawson's findings, also lend support to such a view.

The apparently greater sensitivity to light of the distal portion of a tentacle does not mean that the photoreceptors are most abundant in this region. Rand (1909) and Parker (1917) showed that in the tentacles of *Condylactis,* an actinian, conduction over nerves was always proximal. This supported the histological results on *Cerianthus* of Grošelj (1909), who found that almost all of the sensory cells of the tentacles were unipolar, with their fibrils extending without exception toward the base. Such a situation in the tentacles of *Terebella* would account for the apparently greater sensitivity of the distal portion of the tentacle, as a stimulus applied near the tip would have a greater effect on the tentacle, as a whole, than one applied near the base.

That the direction taken by the majority of fibers from receptors is toward the base is evidenced by the response to electrical stimulation. The similarity, in this respect, to the situation in the tentacles of actinians is of considerable interest. The polarity of the tentacle as regards conduction indicates that a nerve plexus or net probably does not exist here. This is in agreement with the recent work of Coonfield (1932) on earthworms.

SUMMARY

1. The tentacular filaments of *Terebella magnifica* W. are remarkably independent in their behavior after removal from the worm. Their functions are varied and they possess many features typical of an intact organism.

2. A deep ciliated groove acts as a collector and conductor of food to the body of the worm. The epidermis contains numerous specialized mucous cells.

3. The nervous system of the tentacle consists of five longitudinal bundles of nerve fibers with circular connectives or branches occurring at regular intervals.

4. Both intact and isolated tentacles are sensitive to light and in either case the reaction time over a fairly wide range of intensities is of the order of 0.5 second.

5. Local electrical stimulation elicits a response of only that portion of the tentacle proximal to the electrodes except when stimulating with rapid shocks of high intensity.

6. The experimental evidence indicates that the nervous system consists of direct receptor-effector elements which conduct primarily toward the base of the tentacle. There was no evidence which would seem to indicate the presence of a nerve plexus or net.

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THE CONCENTRATION OF EOSIN AND THE PHOTODYNAMIC EFFECT ON TENTACLES OF A TEREBELLID WORM

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An attempt to modify the sensitivity to light of the tentacles of *Terebella magnifica* Webster by means of various dyes occasioned the use of eosin, and necessitated an investigation of its toxicity in the presence of light. The results appear to be of sufficient interest to warrant their separate consideration.

The photodynamic action of light has occupied the attention of numerous investigators since it was first reported by Raab in 1900. Reviews of the literature by von Tappeiner (1909), Clark (1922), and Blum (1932) obviate the necessity of considering the subject historically. The question of immediate interest, that of the effect of the concentration of the dye, has been considered by Fr. von Tappeiner (1908), Pereira (1925), Dognon (1928), and others, but the results in experiments on living systems are for the most part incomplete or qualitative.

Materials and Methods

The tentacular filaments of *Terebella magnifica* W. provide excellent material for such studies. Their general structure and behavior to light has been discussed in an accompanying paper (Welsh, 1934). After removal from the worm the tentacles survive for several days in sea water and when illuminated continue to move about, coiling and uncoiling, as when intact. This constant activity is of considerable importance as it stops rather suddenly at the time of death and provides a definite end point for judging the time of killing. The relative transparency of the tentacles also permits the penetration of a large part of the incident light.

Several fluorescent dyes were employed in the investigation but the results from the use of only tetrabromfluorescein or eosin Y will be considered in this account. The eosin used was a product of the National Aniline and Chemical Co., Shultz No. 587, having a total dye content of 89 per cent. This was made up in sea water to give a one per

cent stock solution by total weight. This stock solution was guarded against exposure to bright light. After a number of preliminary experiments the eosin was used in the following final dilutions; ½, 1, 5, 10, 15, 20, and 25 drops of stock solution to 25 cc. of sea water. It is unfortunate that the dilutions were not made in some other manner, as it is possible to calculate the normality only roughly; but the fact that care was used to control the drop size gave concentrations which for the purposes were sufficiently constant.

The several dilutions of eosin were placed in flat-bottomed glass dishes, 47 mm. in diameter, which gave a 15 mm. depth of solution. This thickness of the layer of eosin in sea water was a nearly constant factor, as the tentacles rested at the bottom and their movement exposed all surfaces to light of the same intensity. Sunlight was used as a

TABLE I

Killing-time in minutes for four sets of tentacles at several concentrations of eosin. (Concentration = drops of 0.89 per cent solution to 25 cc. sea water.) Temp. 32° C. ± 1°. Experiments performed in sunlight, near midday.

Concentration eosin	Time for killing				Av.
	min.	*min.*	*min.*	*min.*	*min.*
Control	—	—	—	—	—
½	60	80	50	35	56.2
1	36	35	40	30	35.2
5	28	25	22	22	24.2
10	20	18	20	20	19.5
15	23	22	25	24	23.5
20	26	25	30	27	27.0
25	30	28	32	31	30.2

source of illumination and the results reported were obtained from experiments performed on cloudless days, near midday, when the rays were essentially perpendicular. The tentacles were exposed to light immediately after being placed in the solutions.

The small containers with the eosin and tentacles were placed in a white enamelled pan and surrounded with water in order to maintain a fairly constant temperature. This averaged about 32° C. Such a temperature was somewhat higher than that normally experienced by the worms but did not appear detrimental to the controls which were run in sea water with each experiment.

RESULTS

Upon exposure to light the tentacles in all of the several dilutions of eosin exhibited greater activity than the control in sea water. They

would coil tightly and then rapidly uncoil. This continued until the epidermal cells began to plasmolyze, when the tentacles straightened, exhibited twitching movements, and soon ceased all activity. This cessation of movement was used as the endpoint and although it did not necessarily indicate the complete death and destruction of all the cells, it marked a point beyond which muscular movement did not occur. It was soon evident that this point was first reached at a concentration of ten drops of stock solution of eosin to 25 cc. of sea water. As the concentration of eosin increased or decreased in relation to this solution the killing-time increased.

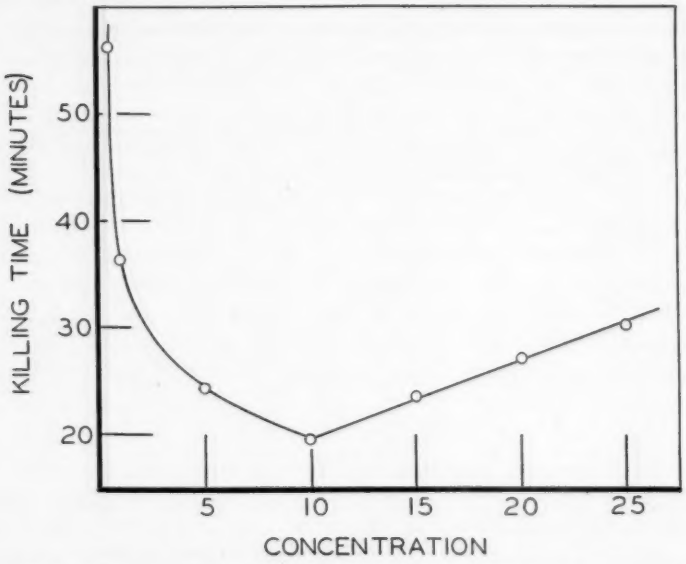

FIG. 1. The average times for killing, given in Table I, are shown plotted against the concentration of eosin. Concentration = number of drops of an 0.89 per cent solution of eosin in 25 cc. of sea water.

Table I gives the times for killing, at the several concentrations, for four separate experiments. In no instance was the control noticeably affected during the exposure although a period of illumination of several hours resulted in an earlier death of the tentacle than if the same were kept in diffuse light. It should also be mentioned that, in the diffuse light of the laboratory, tentacles would live for at least three days in the concentration of eosin which resulted in most rapid killing in direct sunlight. The averages of the times for killing are shown graphically in

Fig. 1. At the lowest concentration the average time for killing was 56 minutes. The times decreased regularly to the 10 drop concentration at which the variations were small and the average killing-time 19.5 minutes. A smooth curve may be drawn through the points obtained and the curve would approach more closely the abscissa were it not for a secondary process entering in which causes an increase in the killing-time. The secondary process is unquestionably due to a shielding effect resulting from an absorption of light by the overlying layer of eosin. It probably follows Beer's Law for the absorption of light. Exceptions to this law are known, but in general the absorption of light by a solution depends on the molecular concentration if the thickness of the absorbing layer is kept constant. This screening effect, which begins to reduce the photodynamic action of the eosin, results in an increase in killing-time and the averages now bear a linear relationship to the concentration over the range employed. If the thickness of the layer of eosin were varied, it is probable that the relationship between concentration and killing-time at the higher concentrations would vary considerably. However, another variable, that of the diffusion of oxygen, would enter in to complicate the matter, as the importance of oxygen in photodynamic processes has been repeatedly demonstrated.

The data of Fr. von Tappeiner (1908) obtained from a study of the effect of concentration of eosin on the hemolysis of red blood cells are in essential agreement with the above. A minimum time for hemolysis of 17 minutes was found at a concentration of 1/2000 N eosin. At concentrations above and below this the times increased. At a concentration of 1/40,000 N the time had increased to 44 minutes and at the highest concentration of 1/200 N the time for hemolysis was 34 minutes. The observations of Pereira (1925) on the combined toxic action of eosin and light on *Arbacia* eggs, sperm, and larvae were qualitative only, but indicated a definite effect of concentration of the dye. Dognon (1928) studied the effect of the concentration of several fluorescent dyes on the killing of paramecia and obtained a definite relationship between concentration and killing-time although he employed only concentrations below that which produced killing in a minimum time.

Results by Gros (1901) on the rate of bleaching of fluorescein in light indicate that at a given concentration the rate of bleaching is at a maximum and that increase or decrease in concentration produces a decrease in rate of bleaching. Blum (1932) mentions other such experiments on non-living systems, all of which yielded essentially similar results.

It would be of interest to employ screens of eosin varying in con-

centration, while keeping tentacles in a given concentration, in order to separate the absorption effect from the photodynamic action. Results obtained in this manner should yield to more precise analysis and permit the formulation of significant mathematical equations to explain the combined effect.

It is evident from the results obtained that the concentration of the fluorescent material is a limiting factor in determining the rate of a photodynamic process. When all other factors are constant the photodynamic effect increases with the concentration of the dye up to a certain point, beyond which there is a decrease due to the absorption of light by the layer of dye. Over the range of concentrations employed in the present study the times for killing, above the minimum, are apparently directly proportional to the concentration of the sensitizing material. Over a wider range of concentrations this relationship probably does not hold, as appears to be indicated by other similar investigations.

Summary

The tentacles of *Terebella magnifica*, W. are satisfactory for the study of certain photodynamic phenomena. The toxic effect of eosin in the presence of light depends upon the concentration of the dye. As the concentration increases, the killing-time decreases to a minimum and then increases linearly over a certain range. This secondary increase is probably due to absorption of light by the overlying layer of eosin.

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THE MOBILIZATION OF CALCIUM DURING DEVELOPMENT

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I

According to Plimmer and Lowndes (1924) (Needham, 1931), the shell membrane of the hen's egg contains only a negligible amount of calcium before the seventh day of incubation. After that the quantity increases sharply and reaches its high point in the neighborhood of 8 mg. CaO on the twenty-first day. In what form is this calcium present?

It was suggested by Tangl (1908) that the embryo eliminating CO_2 itself created conditions progressively more and more favorable to the transformation of the insoluble $CaCO_3$ of the shell into the soluble bicarbonate. A working model of this idea was made by Buckner, Martin, and Peter (1925), who passed a stream of carbonated water through an empty eggshell and demonstrated not only the presence of $CaHCO_3$, but the free diffusion of the bicarbonate from the shell through the adhering shell-membranes. It was inferred "that during the first 9 days of incubation, before the allantois had touched the shell membrane, a water solution containing carbon dioxid given off by the embryo diffuses through the white and membranes to the shell, there forming calcium bicarbonate which diffuses back to the embryo, where it is metabolized. After the ninth day, when the allantois touches the shell membranes, it is reasonable to believe that the shell then gives up calcium bicarbonate to the blood stream as it discharges carbon dioxid."

Is calcium bicarbonate demonstrable in the shell membrane? When the shell membranes were removed from six unincubated Rhode Island Red eggs, and boiled in CO_2-free distilled water, the vapors produced no visible precipitate in $Ba(OH)_2$. Such membranes are not calcium-free, but if they harbor any as bicarbonate its concentration is too low to be detected in this way. However, when this experiment was repeated with the same number of membranes from groups of eggs incubated respectively for 7, 14, and 19 days, precipitates of $BaCO_3$ were formed, and, as might be expected, were progressively more voluminous. For our purposes this qualitative test is sufficient as it harmonizes com-

pletely with the implications of Plimmer and Lowndes (*loc. cit.*) and of Buckner, Martin, and Peter (*loc. cit.*).

II

Since $CaHCO_3$ is present in the shell membranes after one week of incubation, Ca must leave the shell.

The first convincing proof that the shell actually loses Ca, was secured on Plymouth Rock hens by Tangl (*loc. cit.*) and Hammerschlag. These investigators found the following average differences between eight unincubated eggshells and fifteen shells left after hatching. (See Table I.)

TABLE I

*Plymouth Rock Eggshells (with Shell Membranes)**

	Before incubation		After hatching		Loss
	per cent	*grams*	*per cent*	*grams*	*grams*
Dry weight		5.76		5.32	0.440
Organic substance	46.43	2.674	46.06	2.450	0.224
Calcium	37.19	2.142	37.14	1.976	0.166
All else	16.38		16.80		

* Since the analyses of the incubated shells include some Ca removed from the shell but retained by the shell membranes, Tangl's value of 166 mg. for the Ca-loss of the shell itself is probably too low. On the basis of Plimmer and Lowndes' figures (*loc. cit.*) a correction of 4.5 mg. seems justified.

Tangl (*loc. cit.*) comments on the constant relation between organic substance and calcium and suggests the possibility that the embryo derives calcium from organic and inorganic sources in the proportion in which such compounds are present. Referring embryonic Ca accession to $CaCO_3$ lost by the shell accounts for only half the calcium gained during development by the chick and its foetal membranes.

Neither Tangl nor Needham (*loc. cit.*) comment on an inconsistency in these data. If, as the analyses show, the Ca-organic-substance ratio remains constant throughout development, one would expect the same constancy in that part of the shell system which is dissolved. However, the 440 mg. of shell that have disappeared seem to have contained 37.73 per cent of Ca, and 50.45 per cent of organic substance. Is this an accumulation of experimental errors or is the absorption of organic substance and calcium differential?

According to these analyses, total shell weight decreases during incubation, but the concentration of calcium remains almost constant.

III

To verify this percentage constancy on individual eggs requires methods somewhat different from those previously employed. The eggs

we used were all laid during March and April by one Rhode Island Red hen, controlled as to diet and kept monogamous. These eggs were incubated at 39.5° C. in an atmosphere sufficiently moist to insure the highest rate of Ca-metabolism (Romanoff, 1929). After 7 days in the incubator a bit of shell was removed with some assurance that this particular egg would continue normal development at least up to the nineteenth day (Romanoff, 1931). Inasmuch as very little Ca is absorbed by the embryo during the first week of incubation our twelve-day period includes all except one or possibly one and one-half of the significant days. On the assumption that spiral descent in the oviduct assures a uniform concentration—not amount—of calcium in various parts of the shell, one should be able to secure reliable values for the percentage of Ca present in a given eggshell on the seventh day of incubation and

TABLE II

Concentration of Ca in Rhode Island Red eggs. Shells without membranes.

Egg	After 7 days incubation				After 19 days incubation			
	Dry wt. sample	CaO	Ca	Ca	Dry wt. sample	CaO	Ca	Ca
	mg.	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*mg.*	*per cent*
1	78.9	43.3	24.0	30.4	312.6	169.7	94.3	30.2
2	65.2	35.4	19.7	30.2	510.6	272.6	151.5	29.7
3	120.6	64.0	35.6	29.5	826.7	438.6	243.7	29.5
4	144.5	79.4	44.1	30.5	851.3	464.5	258.1	30.3
5	184.1	100.1	55.6	30.2	1516.0	814.5	452.6	29.9
6	188.2	105.0	58.4	31.0	1284.0	691.5	384.2	29.9
				Av. 30.6				Av. 29.9

again on the nineteenth day. It is desirable not to injure the shell membranes and essential to control evaporation. This was done by covering the exposed shell membranes with a layer of lens paper kept continuously moist and held in place by a bit of paraffin. Six individual eggs were studied successfully in this way. The Ca was determined as CaO by adapting the method given in Blasdale (1928). The results, including reductions to Ca, are given in Table II.

Thus individual eggs when compared on the seventh and nineteenth days of incubation also yield essentially constant percentages of calcium.

Since the eggshells lose weight during incubation, this substantial constancy in the concentration of calcium implies an actual removal of Ca from the shell. The quantity mobilized can be computed from the preceding and other data.

The unincubated Rhode Island Red shell has an average dry weight in the neighborhood of 4.36 grams (cp. Needham). Assuming a common rate of Ca-metabolism in all hen's eggs that hatch under standard conditions in 21 days (Byerly, 1932), our shells at the end of the incubation period should have weighed close to 4.03 grams. On the seventh day of incubation the Ca indicated by our analyses is equivalent to 1.334 grams per shell; on the nineteenth day, to 1.205 grams. The loss of calcium from the shell itself is therefore of the order of 129 mg. Applying a conservative 2 per cent correction for the missing days in our experiment (cp. Needham, *loc. cit.*) and correcting Tangl's value for Ca retained in the shell membrane, we arrive at the following orders of magnitude:

Ca lost by Rhode Island Red eggshell ..... 131 mg.
Ca lost by Plymouth Rock eggshell ..... 170 mg.

IV

If the suggestion of Buckner, Martin and Pepper (*loc. cit.*) is correct and allantoic contact with the shell membranes actually enables the shell to give "up calcium bicarbonate to the blood as it discharges carbon dioxid," we should find definite evidence of such erosion.

In order to secure ocular proof, eggs were incubated for 20 days in fixed position. In this way, by keeping each blood vessel constantly at work on the same portion of the shell, we hoped to produce visible erosion grooves. To identify these, we illuminated the eggs before opening and traced the pathways of the major vessels with pencil on the outside.

Although the inside of a shell incubated for 20 days is distinctly rougher than the same surface in a comparable newly-laid egg, convincing identifications of the pathways traced on the outside and the suspected erosion grooves within could not be made by this method alone. We therefore resorted to staining. Other things being equal, a penetrating stain should enter most rapidly where the shell is thinnest. After several failures we tried an alcoholic solution of methyl red. The shells were dipped into the solution, care being taken that no dye could reach the inner surfaces directly. After a considerable amount had penetrated through the shells, these were dried, and compared with unincubated shells treated in the same way. In this manner one can uncover, at times, in unincubated shells certain irregularities in thickness; however, the irregularities in the incubated shells form a branching and anastomosing system and the major ones corresponded to the light pencil-tracings made on the outside before the eggs had been opened. These differences are illustrated in Figs. 1 and 2.

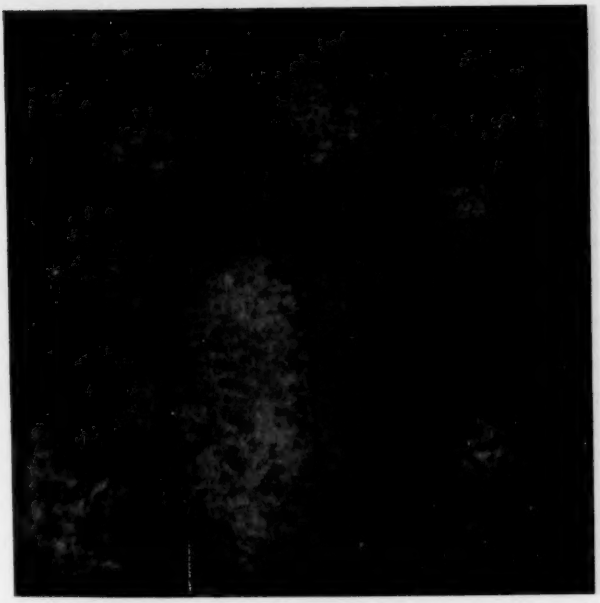

FIG. 1. Inside surface of unincubated eggshell. Treated with methyl red. Magnification about 5 ×.

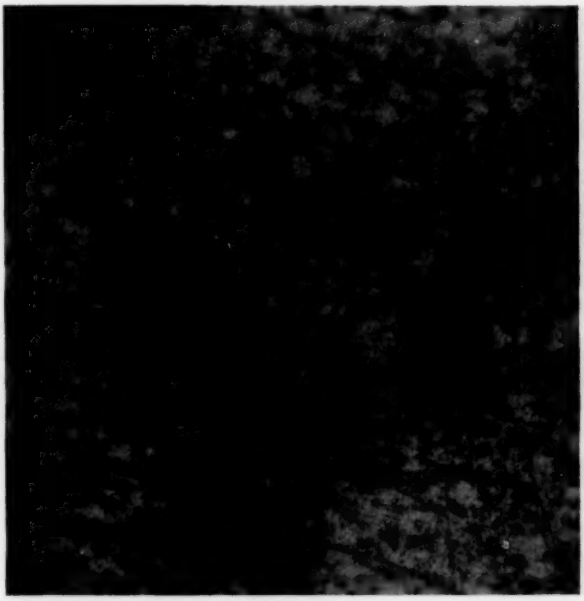

FIG. 2. Inside surface of incubated eggshell. Treated with methyl red to expose the erosion grooves left by allantoic blood-vessels. Magnification about 5 ×.

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NEMA PARASITES IN EMBRYO BATS[1]

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In examining a series of embryos of one of the phyllostomid bats, it was discovered that many of them were parasitized by nematode larvæ. Since infection at such an early stage seems to be almost or entirely unknown, a brief description of the circumstances may be of interest.

The bats in question are *Glossophaga soricina soricina* Pallas, of the South American family Phyllostomidæ; they were collected in November and December, 1933, at Arapuá in eastern Matto Grosso. The material available for study ranges from tubal ova to 15 mm. embryos. The early development is somewhat like that of the European bats. The ovum burrows completely into the uterine mucosa, so that the thick trophoblast is completely shut off from the uterine cavity by a decidua capsularis. The large yolk sac is formed early, before the amnion cavity and embryonic shield are established by the vacuolization of the ectodermic knob. No nemas have been observed in the uteri with the earliest stages. After the blastocyst has begun to expand, however, most of the ova show from one to three parasites located in either the yolk sac or the amniotic cavity. Table I gives the data.

Although all of the parasites found are larvæ, the ones in the older embryos are definitely more advanced in size and organization than those in the younger blastocysts. Most of them are spirally coiled, which makes it difficult to get an accurate measure of their size; fairly accurate measurements of one in embryo No. 73 gave 1.5 mm. as the length. They are not attached to embryo or membranes, but lie free in the extra-embryonic cavities. It is possible, of course, that they were attached and were dislodged by the fixing fluid, but the embryos show no trace of any injuries referable to an attachment of the parasites. In one case discussed below (No. 67) the parasites showed granules in the intestine that looked like the remains of ingested red corpuscles.

There are two conceivable routes of infection by the worms. There is a possibility that the larvæ come from the uterine cavity, boring

[1] The collection and study of this material were made under a Fellowship from the National Research Council, all the work except the actual collecting being done in the Anatomical Laboratory of the Harvard Medical School.

[2] National Research Fellow.

through decidua capsularis, trophoblast, and entoderm into the yolk sac. This does not seem likely, as no parasites have been seen in uteri or tubes, although they would be readily detected in the cavities of these organs. The probable source of the nemas is the maternal blood stream, from which they would make their way through the placental tissues into the cavity of the ovum. There is one observation that seems to give direct support to this idea. In embryo No. 67 the entoderm and trophoblast covering one of the placental vessels (maternal) have been ruptured, so that the yolk sac is half filled with blood, and

TABLE I

Embryo	Stage	No. of nemas	Location
1B 66	Solid ectodermal knob	0	—
1B 39	Amniotic cavity and embryonic shield	1	Yolk sac
1B 60	"	1	" "
1B 79	"	2	" "
1B 67	"	2	" "
1B 51	Primitive streak	2	" "
1B 82	" "	1	" "
1B 64	" "	0	—
1B 69	Neural folds	4	3 in yolk sac 1 between amnion and chorion
1B 80	" "	1	Yolk sac
1B 50	6 somites	0	—
1B 47	2.5 mm. embryo	1	Amniotic cavity
1B 38	2.7 mm. "	1	" "
1B 73	3.6 mm. "	1	" "
1B 42	4.0 mm. "	0	—
1B 54	6.5 mm. "	0	—
1B 85	10.0 mm. "	0	—

among the extravasated corpuscles are two nemas, the apparent agents of the injury. In one or two other vesicles a few maternal corpuscles were seen scattered in the yolk sac, but in most cases the penetration of the larva does not seem to cause bleeding, the tissues closing without leaving any trace of the worm's passage. At the time when the parasites bore through into the blastocyst, the yolk sac cavity fills almost all the vesicle, consequently this is where the nemas are usually found (but see No. 69 in Table I). Later, as the amnion cavity expands and the lumen of the yolk sac is reduced to a very narrow slit, the worms seem to migrate through yolk sac and amnion into the cavity of the latter.

No evidence is available to show the ultimate fate of the parasites.

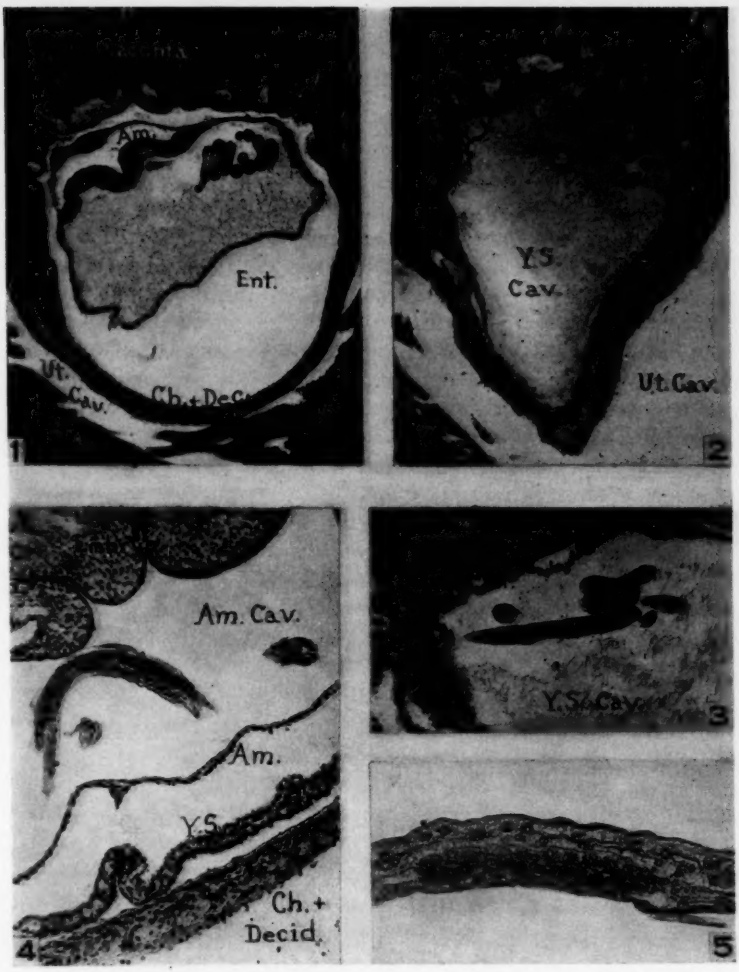

FIG. 1. Embryo 60. Section through amniotic cavity, embryonic shield, and yolk sac, showing a nema coiled up in the cavity of the yolk sac. The space between entoderm and chorion is due to shrinkage. × 60.

FIG. 2. Embryo 67. Section through yolk sac. Note the broken entoderm at the top, apparently caused by the two nemas, a few coils of which show in this section. The granular material surrounding them is blood, introduced by the migration of the parasites from the maternal blood vessels. × 60.

FIG. 3. Embryo 69. A nema in the yolk sac. × 120.

FIG. 4. Embryo 47. Three sections of a nema lying in the amniotic cavity. × 120.

FIG. 5. Part of a nema from embryo 38. Cuticle, ectoderm, and gut can be seen, as well as the elongated, darker-staining gonad. × 240.

Abbreviations: *am.*, *am. cav.*, amnion, amnion cavity; *ch.*, chorion; *decid.*, decidua capsularis; *ent.*, entoderm; *ut. cav.*, uterine cavity; *y. s.*, *y. s. cav.*, yolk sac, cavity of yolk sac.

In the oldest embryos (3.6 mm.) in which they have been found, the worms are still in the amniotic cavity. It is possible that they may persist here and so be lost with the discarding of the extra-embryonic membranes at birth. If so, their presence in the vesicles would have to be looked upon as being without significance. On the other hand, they may find their way into the embryo itself at some time before birth and so survive, in which case we would be dealing with a definite mechanism for the transmission of the parasite from mother to fetus. The high percentage of infection (65 per cent in the series examined) would seem to indicate that something more than mere accident is involved, but a study of a series of adults and of newly born individuals will be necessary before definite conclusions can be drawn.

It has not been possible to identify the larvæ up to this time. The slides have been examined by Dr. J. H. Sandground, of the Department of Tropical Medicine, who tells me that the worms are too immature to identify with certainty but that they may be the larvæ of some strongylid nematode. Nemas from the cœloms of some of the parent bats have been studied by Dr. Sandground and pronounced a new species of the filariid genus *Litomasoides;* they thus have no relationship to the parasites found in the embryos. I extend here my thanks to Dr. Sandground for his assistance in this phase of the investigation.

THE INFLUENCE OF HYPERTONIC AND HYPOTONIC SEA WATER ON THE ARTIFICIAL ACTIVATION OF STARFISH EGGS

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In the previous paper (2) the general conditions of artificial activation in the starfish egg were summarized briefly as follows: (*a*) a non-toxic penetrating acid (*e.g.* a fatty acid) acting alone, either in the presence or absence of oxygen, activates the egg completely and at a rapid rate which is closely proportional to the cH of the acid solution[1]; (*b*) hypertonic sea water may also cause complete activation but at a much slower rate and only in the presence of oxygen.

The important fact is that either of these two quite different changes of condition may produce the same physiological effect; but apparently they do so by inducing or promoting within the egg biochemical reactions of dissimilar kinds.[2] The hypothesis was proposed that the acid acts by catalyzing the hydrolytic decomposition of some ester-like compound (possibly phosphagen) normally present in the egg, and that the reaction which directly determines activation consists in the chemical union of a product of this hydrolysis with some other compound also present or available in the egg. This second compound appears to be formed (or its quantity increased) under the influence of hypertonic sea water in the presence of oxygen, the presumption being that the abstraction of water promotes its dehydrolytic synthesis from certain pre-

[1] Heat activation is regarded as a special case of acid activation.

[2] Certain other characteristic differences in the physiological effect of the two treatments are in harmony with this conclusion. Thus, if the eggs are exposed briefly to heat (32°–35°) or acid (e.g., .004m butyric acid in sea water) within a few minutes (up to ten) after removal from the starfish, they not only show no activation but remain permanently immature with germinal vesicle intact (4). Hypertonic sea water, on the contrary, activates the eggs in a typical manner when the exposures are begun during this period. The immediate effect produced by the hypertonic sea water is apparently independent of the breakdown of the germinal vesicle; while the acid is quite without activating effect until this change has occurred, after which the egg is readily activated by both heat and acid. Apparently the substance which reacts with the acid is either absent or unavailable until the germinal vesicle has broken down.

cursors. The combination of the two compounds produces a third compound, the activating substance, whose accumulation renders the egg capable of automatic development.

According to this hypothesis the critical change determining activation is the chemical union of the hydrolytic product, which we designate as B, with the synthetic product, called S, to form the activating substance, A. This reaction (activation reaction) would then be represented by the equation

$$S + B = A,$$

and its velocity by the equation

$$V = K\ C_a C_b,$$

where K is the velocity constant of the reaction, and C_a, C_b the concentrations of S and B respectively.

B is regarded as being freed by the activating acid at a rate proportional (through a certain range) to the cH; it combines as rapidly as it is formed with S; hence the proportionality between the cH and the rate of activation. In normal untreated eggs the concentration of S (*i.e.*, C_a) is assumed to be a constant, fixed by the inherited constitution of the egg. With C_a thus constant, the rate of activation should be proportional to the concentration of the activating solution of acid, as we find (3). If, however, we vary C_a, as by previously treating the eggs with hypertonic sea water, the rate of activation in a constant solution of acid should vary correspondingly; under such circumstances this rate, according to the formula, would be proportional to C_a.

Experiment shows that unfertilized eggs treated for a brief period (5–40 minutes) with hypertonic sea water and then exposed to acid show the usual course of acid activation, but at an increased rate (Table I). This characteristic effect may be described by saying that the eggs are *sensitized* to the activating influence of the acid.[3] According to our hypothesis the effect follows from the dehydrolytic synthesis of additional S (called also the sensitizing substance). In this synthesis a number of simple molecules are assumed to interact to form a molecule of the more complex compound S; in the successive steps of any such synthesis the molecules are to be conceived as combining successively in pairs; and the rate of the total synthesis will be determined by the rate of interaction of the pair of molecules which combine most slowly.[4]

[3] The analogy with the sensitization of photoreceptors to light during the process of dark-adaptation, which follows a very similar time course (6, 7), is pointed out below.

[4] The relation of oxygen to hypertonic activation suggests that the combination of molecular oxygen with some other compound may be the controlling reaction.

The course of hypertonic sensitization would then be expected to follow the curve of a bimolecular reaction. In the following experiments the rate of activation by a constant solution of butyric acid, .004M in sea water, has been determined after exposing the eggs for varying periods to hypertonic sea water of constant composition.

Action of Hypertonic Sea Water Followed by Acid

A typical experiment is summarized in Table I. In all experiments of this kind the eggs were placed, shortly after the breakdown of the germinal vesicle, in hypertonic sea water of a constant composition (100 volumes sea water *plus* 20 volumes 2.5 M NaCl). After varying intervals, usually 10, 20, 30 and 40 minutes, portions were transferred to normal sea water, where they remained 10 or 15 minutes; they were then transferred to .004 M butyric acid solution in sea water. From this solution portions were returned to normal sea water at regular intervals (usually of 1 minute between 1 and 10 minutes). The approx-

Table I

July 7, 1932. Eggs from several animals were removed at 3:40 and placed in hypertonic sea water at 4:10. Temperature of solutions and sea water 22°. In the fertilized control practically all eggs developed normally.

In hypertonic sea water (22°)	Interval in normal sea water	Percentages of eggs forming blastulæ after exposure to .004 M butyric acid (22°) for the times indicated (min.)								
		(Control: hyp. sea water alone)	2	3	4	5	6	7	8	9
min.	*min.*									
A. 0*			0	pr. 0	15–20	40–50	70–80	80–90	70–80	50–60
B. 10	10	0	pr. 0	*ca.* 1	30–40	*ca.* 90	70–80	*ca.* 50	30–35	*ca.* 10
C. 20	12	0	<1	*ca.* 20	55–65	60–70	*ca.* 50	*ca.* 5	0	0
D. 30	12	pr. 0	*ca.* 1	25–30	70–75	50–60	30–40	3–5	0	0
E. 40	12	<1	5–10	40–50	65–70	*ca.* 50	15–20	*ca.* 5	0	0

* Control: acid alone.

imate percentages of eggs developing to blastulæ were later determined. Controls of eggs exposed to hypertonic sea water without acid treatment, and to butyric acid without hypertonic treatment, were always kept.

In this experiment the series treated with butyric acid alone (*A*) shows the typical increase in the proportion of favorably developing eggs with increasing time of exposure up to a definite optimum at about 7 minutes, after which there is a decline. With the eggs treated previously with hypertonic sea water a similar time curve of activation is

seen in each of the series *B* to *E*, but the effective exposures (as measured by the formation of blastulæ) are in all cases briefer, and the more so the longer the treatment with hypertonic sea water. The optima are respectively *A*, 7–8 minutes; *B*, 5 minutes; *C*, 4–5 minutes; *D*, *ca.* 4 minutes; *E*, *ca.* 4 minutes.

In ten experiments of this kind in June and early July a similar result was found. In two of these (June 10 and June 11, 1931) the temperature of the solutions was 18° and the optimal exposures to the acid alone were longer (*ca.* 20 minutes). The other eight experiments are summarized in Table II; these were carried out under closely similar conditions; the temperature of the hypertonic sea water and butyric acid solution was 20°–22°, and the optimal exposures to the acid alone varied between 7 and 9 minutes. The table gives the results of exposures to butyric acid after treatment with hypertonic sea water (100 + 20) for periods varying from 10 to 40 minutes. The single experiments are in good agreement, and the averages show a regular decrease of activation-time with increase in the duration of the hypertonic treatment. Up to 40 minutes few or no eggs develop to blastulæ as a result of the hypertonic treatment alone; but an exposure of 60 minutes to hypertonic sea water alone usually activates a considerable portion of eggs, so that the sensitizing effect then becomes difficult to estimate. In the experiments of June 17 and June 18 eggs exposed to hypertonic sea water for 60 minutes formed a considerable proportion of blastulæ without further treatment (*ca.* 10 per cent), and 50 per cent or more blastulæ with additional exposures of 1 and 2 minutes to butyric acid.

The data show that the action of hypertonic sea water in shortening the effective exposures to butyric acid is most rapid during the earlier part of the immersion and falls off regularly as time advances. In Fig. 1 the average optimal exposures to acid (optimal activation-times) are plotted against the times in hypertonic sea water. The curve drawn is the theoretical bimolecular isotherm, the assumptions being (1) that the rate of the activation-process (the reciprocal of the activation-time) is proportional to the concentration of *S*, and (2) that the rate of formation of *S* is controlled by the interaction of two precursor molecules of equal concentration ($P + Q = S$). The simple bimolecular formula is used,

$$\frac{1}{t} \cdot \frac{x}{a(a\text{-}x)} = K;$$

a represents the initial concentration of *P* or *Q*, and *x* the concentration of *S* formed by the time *t*. The initial concentration, *a*, is equal to the difference between the concentration of *S* when the reaction is complete,

TABLE II

Optimal exposures (minutes) to butyric acid solution (.004 M in sea water at 20–22°) and percentages of eggs forming blastulæ (in brackets) after previous treatment with hypertonic sea water for varying periods.

Experiment no.	Date	Durations of previous exposure (minutes) to hypertonic sea water (20°–22°)						
		0 (control)	10	15	20	30	40	60
1.	June 17	9 (75–80)			5 (30–35)		4 (30–40)	1–2 (*ca.* 50)
2.	June 18	7 (80–90)			5–6 (*ca.* 50)		4 (*ca.* 50)	1–2 (*ca.* 50)
3.	June 29	7 (*ca.* 50)	5 (35–40)		4 (20–30)	4 (20–30)		
4.	June 30	7–8 (65–75)	6 (50–60)		5 (40–50)	5 (35–45)	4 (35–45)	
5.	July 6	8 (70–80)	5 (55–65)		4 (30–35)	3 (35–40)	2–3 (35–45)	
6.	July 7	7 (80–90)	5 (80–90)		4–5 (55–70)	4 (70–75)	4 (65–70)	
7.	July 8	7 (75–85)		5 (50–60)		4 (*ca.* 50)	3 (*ca.* 40)	
8.	July 11	8 (70–80)	5 (60–70)		5 (*ca.* 50)	3–4 (30–35)	3 (30–35)	
Averages		7.5 min.	5.2 min.	5 min.	4.7 min.	3.9 min.	3.5 min.	

and its concentration at the beginning of the reaction (*i.e.*, before any hypertonic treatment); this difference is regarded as proportional to the difference between the optimal activation-times at these two periods. a-x is the concentration of S yet to be formed at any stage in the reaction; *i.e.*, when the latter is complete a-x becomes zero. S is steadily being formed during the immersion in hypertonic sea water but at a progressively decreasing rate. The time required for complete transformation of the precursors into S is estimated by the extrapolation of the curve to its asymptote or final level. This time is estimated at about 90 minutes, and the limiting value of the activation-time as 2 minutes. The calculated curve is drawn on this basis (see Table III), and the position of the points representing the observations shows a good correspondence with the theory.[5]

TABLE III

The final concentration of S is regarded as that corresponding to an optimal activation-time of 2 minutes. The value of a is then 7.5 − 2.0 = 5.5. The additional concentrations of S formed after the times t are regarded as proportional to the decreases in the optimal activation-times (column 3).

Time in hypertonic sea water (t)	Optimal activation times	Additional S formed at time t (x)	P or Q at time t (a–x)	K	Activation-times calculated from average value of K
min.	*min.*				*min.*
0	7.5	0			
10	5.2	2.3	3.2	0.013	5.36
20	4.7	2.8	2.7	0.010	4.43−
30	3.9	3.6	1.9	0.0115	3.88
40	3.5	4.0	1.5	0.012 (average 0.0115)	3.56

It is interesting to note that the curve resembles in its general character the curves representing the course of sensitization to light during the process of dark-adaptation, as recently investigated by Hecht. He

[5] The estimated activation-time of 2 minutes corresponding to the maximal concentration of S cannot be determined directly, for the reason that after sufficient exposure to hypertonic sea water many eggs develop completely without any acid treatment. This has already occurred at 60 minutes in the two experiments of June 17 and 18 (Table II). The optimal exposures to hypertonic sea water alone at 20° are between 75 minutes and 2 hours; this would indicate that maximal sensitization has then been reached. On the average there is not much difference in the effects of exposure between 75 and 120 minutes. Runnström finds that acid is formed in the sea-urchin egg after prolonged exposure to hypertonic sea water (6); such an effect would account for the formation of the hydrolysate B required for the assumed reaction $S + B = A$, and would also explain why after 60 minutes in hypertonic sea water the effect of the butyric acid solution is greater than would otherwise be expected.

regards dark-adaptation as resulting from the accumulation of a light-sensitive compound formed by a bimolecular (*i.e.*, bimolecularly-controlled) reaction, and he has shown that many forms of dark-adaptation follow a curve of this type (7). Hartline has recently shown the same for the eye of *Limulus* (8). Hecht's curve of the light-sensitization of *Pholas* during 2½ hours in the dark (9) may be compared with Fig. 1.

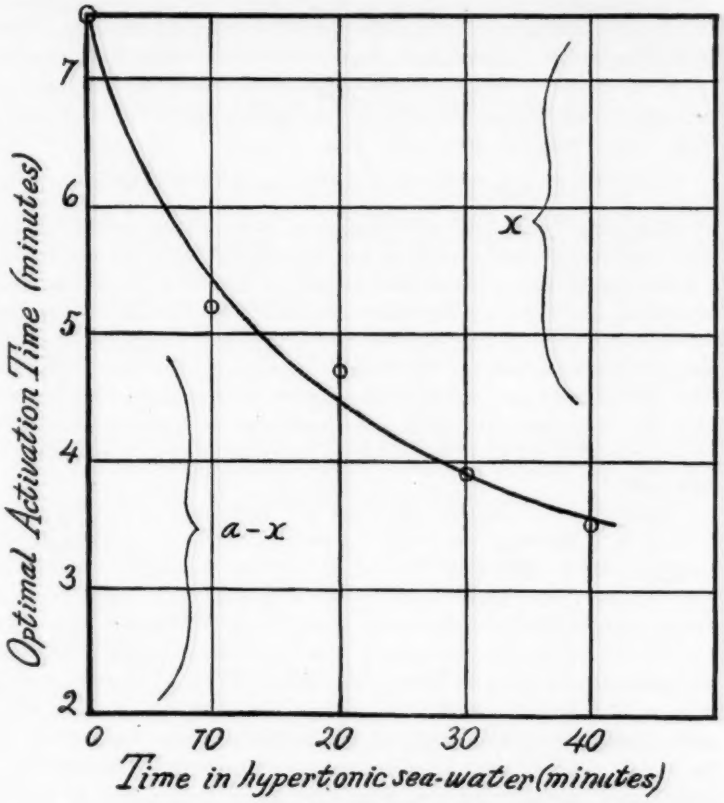

FIG. 1. Ordinates: average optimal activation-times (minutes of exposure to .004 M butyric acid in sea water). Abscissæ: previous exposures to hypertonic sea water (minutes). Temperature 20°–22° (see Table II).

A constant light-intensity (analogous to the constant acid-concentration used in the present experiments) was used for stimulation, and the reaction-time (= activation-time) was found to decrease progressively to a minimum, in a manner resembling closely that just described.

The fact that the sensitizing effect of hypertonic sea water appears so soon after immersion and that the curve shows so steep a descent during the first few minutes, indicates that the loss of water from the site of the sensitizing reaction is rapid and reaches equilibrium almost immediately. Localization of the reaction in the most superficial layer of the egg cortex would thus be indicated, possibly the layer immediately beneath the outer pellicle; the latter is apparently scleroprotein in nature, and is separated on activation as the fertilization membrane (10). This would imply that a more internal layer controls the osmotic loss of water from the egg as a whole, a process requiring apparently about 10 minutes to reach equilibrium, as indicated by the curve of shrinkage in sea water made hypertonic with glycol (11, 12).

Butyric Acid Followed by Hypertonic Sea Water

When a brief exposure to butyric acid, sufficient for partial but not for complete activation, precedes the hypertonic treatment, the results are less regular than with the reverse order. The effective exposures to hypertonic sea water are shortened, but the increase in rate of response to this agent (as measured by the decrease in effective exposures) shows no very definite relation to the time in butyric acid solution. When the exposures to acid are sufficient for complete or almost complete activation, the subsequent treatment with hypertonic sea water is ineffective or injurious; while with longer than optimal exposures it is definitely injurious.

These effects are illustrated by the two series summarized in Tables IV and V. The eggs were placed, shortly after the breakdown of the germinal vesicle (30–35 minutes after removal from the starfish), in .004 M butyric acid in sea water for the times indicated; they were then returned to normal sea water, and after 15 or 20 minutes they were exposed to hypertonic sea water. The percentages forming blastulæ and gastrulæ are given as before; the results of single treatments with acid or hypertonic sea water are also given. Both lots of eggs were normal and responded normally to sperm fertilization. Table IV gives the results observed with briefer exposures to acid, and Table V those with longer exposures ranging from sub-optimal (6 minutes) to supra-optimal (12 minutes).

In fourteen experiments of this kind during the summers of 1931–32 the results were in general similar but with considerable variation in detail. The previous treatment with acid appears to disturb the regularity of the response to hypertonic sea water, possibly because of changes in the osmotic properties of the surface layer; and regular quantitative relations of the kind observed with the reverse order of treat-

ment are difficult to discern. The effective exposures to hypertonic sea water are, however, almost invariably shortened by brief treatment with butyric acid; this effect may be attributed to the formation of the acid-product, *B*.

TABLE IV

Experiment of June 26, 1931. Concentrations of hypertonic sea water and butyric acid solution the same as before; temperature of both *ca.* 20°.

Time in acid solution	Durations of exposure to hypertonic sea water (minutes) and percentages of resulting blastulæ								
	(Control: acid alone)	10	20	30	40	50	60	75	90
min.									
A. 0*		0	0	<1	20–25	30–40	50–60	60–70	65–75
B. 1	0	*ca.* 1	20–25	30–40	40–45	*ca.* 50	40–50	*ca.* 50	
C. 2	0	*ca.* 1	*ca.* 10	40–50	*ca.* 50	50–60	*ca.* 50	30–40	
D. 4	0	*ca.* 1	*ca.* 1	10–15	40–50	*ca.* 50	50+	50–60	
E. 6	15–20	*ca.* 1	*ca.* 1	*ca.* 1	*ca.* 1	*ca.* 1	3–5	3–5	

* Control: hypertonic sea water alone.

TABLE V

Experiment of June 29, 1932. Similar conditions. Temperature 21–22°.

Time in acid solution	Exposures to hypertonic sea water (minutes) and percentages of blastulæ							
	(Control: acid alone)	15	30	45	60	75	90	105
min.								
A. 0*		<1	2–3	*ca.* 50	*ca.* 90	85–90	75–85	60–70
B. 6	60–65	60–70	70–80	60–70	65–75	60–65		
C. 8	90+	50–60	50–60	30–40	30–40	25–35		
D. 10	30–40	*ca.* 5	*ca.* 1	<1	<1	<1		
E. 12	<1	<1	<1	0	0	0		

* Control: hypertonic sea water alone.

HYPOTONIC SEA WATER FOLLOWED BY BUTYRIC ACID

Theoretically we should expect that if hypertonic sea water acts by reducing the concentration of water at the site of the activation reaction, thus shifting the equilibrium in the direction of the further dehydrolytic synthesis of the sensitizing compound, *S*, the action of dilute sea water would be in the opposite direction; *i.e.*, previous exposure to hypotonic sea water should lengthen the activation-time in butyric acid solution.

Experiments were performed similar to those already described, but with hypotonic substituted for hypertonic sea water. The following dilutions of sea water were used: 15, 20, 25, 30, 35 and 40 volumes distilled water in 100 volumes of the mixture (*i.e.*, 85, 80, 75 etc. volumes per cent sea water). The eggs were placed in the hypotonic medium for periods of 15 to 30 minutes (*ca.* 20°), returned to normal sea water for 10 or 15 minutes, and then exposed to .004 M butyric acid in normal sea water.

Eggs treated with dilute sea water and then returned to normal sea water without further treatment show no membrane-formation or other signs of activation. The osmotic intake of water has thus by itself no evident activating action. Previous treatment with slightly dilute sea water (80 to 85 volumes per cent) has in some cases been followed by retardation of acid-activation, but the results have not been uniform. With the higher dilutions (75–60 volumes per cent sea water) the reverse effect was usually seen. This apparent acceleration of acid-activation by the more dilute sea water appears to be a summation effect. The conditions suggest that in the more dilute solutions the hypotonic distension has in itself some activating (acid-producing) effect which may compensate or mask the influence of dilution on the *S*-producing reaction.

Influence of Hypertonic and Hypotonic Sea Water on Activation by Warm Sea Water (32°)

During the course of maturation starfish eggs lose their responsiveness to heat-activation sooner than to acid-activation. After the separation of the first polar body (60–65 minutes after removal in sea water at 20°), eggs exposed to sea water at 32° for the optimal time give few if any blastulæ, while the response to butyric acid remains normal for some time longer. After the second polar division (90–100 minutes after removal), the response to acid declines rapidly. At 2 hours few if any eggs form blastulæ.

The time during which experiments with combined heat and hypertonic treatments can be carried out with fully responsive eggs is thus somewhat abbreviated. Nevertheless, the general type of result is the same as with acid-activation. In eggs treated previously with hypertonic sea water the response to heat is definitely accelerated. This has been established in a large number of experiments, the details of which need not be given. The results have shown somewhat less quantitative regularity than in the case of acid-activation; this difference may be attributed partly to the greater difficulty of controlling the conditions

and partly to variations in the acid-producing reaction within the egg.[6]

The following experiment (July 6, 1933) is typical (Table VI). Eggs were exposed to hypertonic sea water (beginning 30 minutes after removal) for (*B*) 15 minutes and (*D*) 38 minutes, returned to sea water (20°) for a short time, and then exposed to the warm sea water (32°). The controls (*A* and *C*) were treated (at the same interval after removal as *B* and *D*) with warm sea water alone.

With all times of exposure to 32° (from 2 minutes on) activation was more rapid in the eggs treated previously with hypertonic sea water. The eggs in Experiment *C* were exposed after the first polar division and show only slight response; in Experiment *D* maturation has been delayed during the stay in hypertonic sea water and responsiveness remains good at the same interval after removal. Hypertonic sea water has been found uniformly to retard the loss of response to heat-activation, besides decreasing the effective times of exposure.

TABLE VI

Treatment of eggs	Result
A. 32° alone in sea water, beginning 51 min. after removal	Optimum at 6 min.: 60–65% blastulæ
B. Hypertonic sea water for 15 min.; normal sea water 12 min.; then 32° in sea water, beginning 52 min. after removal	Optimum at 4–5 min.: 65–75% blastulæ
C. 32° alone, beginning 80 min. after removal	Optimum at *ca.* 5 min.; few blastulæ (*ca.* 5%)
D. Hypertonic sea water 38 min.; sea water 8 min.; 32° in sea water, beginning 81 min. after removal	Optimum at 4 min.; many blastulæ (*ca.* 90%)

Experiments with hypotonic sea water have so far been few, and the influence on activation times was not constant. In this case also the loss of responsiveness is delayed during immersion in the dilute medium.

Hypertonic Sea Water and Acid Acting Simultaneously

In the experiments so far described, where fatty acid and hypertonic sea water were used in combination, the two treatments were applied successively, with a brief interval in normal sea water. When the treat-

[6] If we regard heat-activation as a special case of acid-activation, it would seem that some reactant or catalyst in the acid-producing reaction within the egg is lost or destroyed near the time of the first polar division. The other substances required for activation by acid (whether the acid is formed within the egg or enters from without) appear to remain unaltered for some time longer, at least until the second polar division, after which the response to all types of activation, including sperm-fertilization, rapidly declines.

ments are applied simultaneously, *i.e.*, when the eggs are exposed to hypertonic sea water containing .004 M butyric acid, a somewhat unexpected result is obtained. The activating action of the acid is markedly retarded, and the period during which this action is exhibited is correspondingly prolonged.

The following experiment will illustrate (June 22, 1933). Eggs of the same lot were treated simultaneously during the prematurational period with .004 M butyric acid dissolved (*A*) in normal sea water and (*B*) in hypertonic sea water, and then returned to sea water. Table VII gives the durations of exposure and the approximate percentages of eggs forming blastulæ.

Eight experiments of this kind were carried out during June 1933, and in all a similar effect was shown, with some variation in degree. The delay in activation appears to be correlated with a delay in the structural change associated with activation. In all experiments mem-

TABLE VII

Times of exposure (minutes at 20.5°) and percentages of blastulæ

Solution used	2	4	6	8	10	12	14	16	18	20	22	24
A. .004 m. butyric acid in normal sea water.....	*ca.* 1	*ca.* 5	50	80–90	*ca.* 80	20–25	<5	0				
B. .004 m. butyric acid in hypertonic sea water.....	*ca.* 1	*ca.* 1	<5	15–20	30–40	70–80	70–80	60–70	*ca.* 60	20	10	0

brane-formation was prevented or retarded, the range of effective exposures was lengthened, and the breakdown following supra-optimal exposures was delayed. In one case 50 per cent of eggs formed blastulæ after an exposure of 24 minutes.

There is much evidence that a structural change in the surface-layer or "cortical zone" of the egg is a constant factor in both normal and artificial activation in many species; this evidence is seen in membrane-formation in echinid eggs, increase of permeability to water and dissolved substances, visible breakdown of the cortical layer in annelid eggs, and similar effects. Conversely, agents which alter the structure of the surface-layer, such as cytolytic compounds, mechanical agitation, and strong electric currents, have an activating effect.[7] The retarding

[7] The fact that the progress of the activation-reaction in warm sea water has such a high temperature-coefficient (1), and that the reaction may be immediately arrested at any stage by returning the eggs to sea water, suggests again a dependence of activation on some readily reversible structural change. Activation

influence of hypertonic sea water on acid-activation, shown in the experiments just described, may be interpreted as an expression of increased structural coherence or density resulting from the partial dehydration of the surface-layer. To the delay in the structural change corresponds the delay in the primary chemical reaction of activation. In the terms of our general hypothesis, the rate of union of the reactants *S* and *B* may be regarded as determined in part by the structural conditions (*e.g.*, permeability conditions) controlling the rates of diffusion at the site of the activation-reaction, as well as by the conditions of concentration already considered. Such a view would account both for the retardation of the reaction and for its being spread out over a longer period of time.

Nature of the Chemical Reactions of Activation

The special nature of the chemical reactions underlying activation is still obscure. In the former paper (2) it was pointed out that certain conditions of acid-activation (such as independence of oxygen-consumption) indicate a hydrolysis or other type of non-oxidative splitting process as the most probable type of primary reaction, and from the analogy with other cells (muscle, yeast, nerve) the splitting of a phosphagen ester was suggested. An attempt to test this hypothesis was made in the summer of 1932, by the analytical estimation of the free phosphate in the eggs before and after fertilization; but the analyses [8] (somewhat few in number) gave no indication of any change. A further corollary of the phosphagen hypothesis is that the other possible hydrolytic products might be concerned in the reaction-sequence of activation; but experiments in which eggs were warmed at 32° (1 to 9 minutes) in sea water containing the phosphagen-forming bases, creatin, arginin, and guanidin (0.1 to 0.4 per cent creatin hydrate, and hydrochlorates of the other bases in similar concentrations) also gave essentially negative results. If such compounds penetrate the egg in the warm sea water, as it seems reasonable to suppose—since (*e.g.*) hydrochloric acid in low concentration gives evidence of penetrating under these conditions (2)—they are without influence in the rate of the activation-reaction. Up to the present, at least, the phosphagen hypothesis has received no support from the experiments which it has suggested.

A similar uncertainty exists with regard to the nature of the acid

arrested in this way can be renewed and carried to completion by a second exposure to heat, or by exposure to acid or hypertonic sea water (1).

[8] Carried out by Dr. Pei-Sung Tang, to whom my best thanks are due. Runnström also has been unable to find any change in the free phosphate of sea-urchin eggs after fertilization (6).

which we have inferred—from the close resemblance between heat and acid-activation—to be formed within the egg during the exposure to warm sea water. If heat-activation is a form of acid-activation, the most obvious supposition would be that lactic acid is formed (within the egg-cortex) by glycolysis, very much as in the heat rigor of muscle. Increase in lactic acid following fertilization has recently been observed in sea-urchin eggs by Perlzweig and Barron (13). I have found, however, that iodoacetic acid, which blocks glycolysis in a large variety of cells, does not interfere appreciably with heat-activation in starfish eggs. In three successive experiments in which eggs (from three different lots) were warmed to 32° in sea water containing 0.02 per cent Na-iodoacetate the course of heat-activation was entirely normal, 60 per cent or more of the eggs forming blastulæ with exposures of 4 to 6 minutes. The only noticeable difference from the control (32° in normal sea water) was a slight shortening of the activation-time in the presence of iodoacetate and the production of a somewhat larger proportion of blastulæ. This result seems quite inconsistent with the glycolysis hypothesis of heat-activation, unless we assume absence of penetration, or that iodoacetate is without influence on the acid-forming reaction in these eggs.[9] The possibility may be considered that some acid other than lactic, *e.g.*, pyruvic, is responsible for heat-activation. Further experimental and chemical work is required.

Summary

Brief treatment of unfertilized starfish eggs with hypertonic sea water increases the rate of activation by subsequent exposure to butyric acid. This effect may be described as a sensitization to the activating influence of the acid. The curve representing the relation between the duration of the hypertonic treatment and the rate of acid-activation resembles the bimolecular reaction isotherm.

In general the experimental facts of acid-activation (and of heat-activation) are consistent with the following simplified schema. Activation is the physiological result of the accumulation of a reaction product, called (provisionally) "the activating substance" (substance *A*) which is formed by the chemical union of two chief compounds. One

[9] Sodium fluoride also, in experiments with .002, .004, .008 and .01 per cent solutions in sea water, was found not to interfere with heat-activation. In one experiment 90 per cent of eggs formed blastulae after 6 minutes in sea water at 32° containing .01 per cent NaF.

Dr. E. S. Guzman Barron informs me that he has found iodoacetate to have no depressant action but, if anything, a slight promoting action upon oxygen-consumption in *Arbacia* eggs.

of these, called (after the analogy with light-sensitization) the "sensitizing substance" (substance S), is already present in the egg; the other, the "acid-product" (substance B), is set free (*e.g.*, by hydrolysis) under the influence of the activating acid. The equation $S + B = A$ symbolizes this hypothetical activation-reaction.

Treatment with hypertonic sea water increases the concentration of S at the site of the activation-reaction, through the abstraction of water and promotion of dehydrolytic synthesis. The resulting increase in the rate of acid-activation is thus explained. Treatment with dilute sea water should theoretically have the reverse effect, but in this case the experimental evidence is conflicting.

The acid-product B appears to be formed at a rate proportional (through a certain range) to the concentration of acid; it is regarded as being set free by the hydrolysis of some normal egg-component at a rate controlled by the cH. The special hypothesis that the hydrolyzed compound is a phosphagen ester has, however, received no support from test experiments.

The hypothesis that heat-activation is an effect of the formation of lactic acid by glycolysis is also not supported by experiments with iodoacetate and fluoride.

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